MOLECULAR STUDIES OF GENES REQUIRED FOR NITRATE ASSIMILATION IN FUNGI AND HIGHER PLANTS

Ghassan Jadou Mousa Kana'n

A Thesis Submitted for the Degree of PhD
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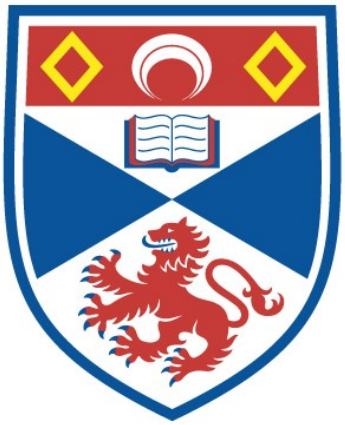

1997

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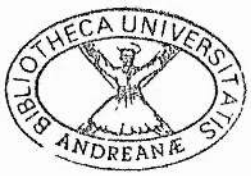
Submitted In Accordance With The Requirements
For The Degree Of Doctor Of Philosophy.



The University Of St. Andrews
School Of Biological And Medical Sciences.
December 1996

DECLARATION

I, Ghassan Jadou Mousa Kana'n, hereby certify that this thesis has been composed by myself, that it is a record of my own work and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Date 13/12/1996

I was admitted to the Faculty of Science at the University of St. Andrews as a Candidate for the degree of Ph.D. in January 1994.

Signed Date 13/12/1996

CERTIFICATE

I hereby certify that the Candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D. at the University of St. Andrews, and that the Candidate is qualified to submit this thesis in application for that degree.

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TABLE OF CONTENTS

CHAPTER TWO

MATERIAL AND METHODS

CHAPTER THREE.

ISOLATION AND GROWTH CHARACTERISATION OF CHLORATE RESISTANT MUTANTS

CHAPTER FOUR

CHARACTERISATION OF *crn* MUTANTS

CHAPTER FIVE

CHARACTERISATION OF NITRITE UPTAKE ACTIVITIES IN *A. nidulans*

CHAPTER SIX

CHARACTERISATION OF THE *cnx* MUTANTS

Abbreviations

A_{204} nm	Absorbance at 204 nm
A_{595} nm	Absorbance at 595 nm
Arg	Arginine Auxotrophic Requirment
APS	Ammonium Persulphate
Bio	Biotin Auxotophic Requirment
bp	Base Pair
BSA	Bovine Serum Albumin
BPB: NR	Bromophenol Blue Nitrate Reductase Activity
Cs	Caesium
CsCl	Caesium Chloide
Conc	Concentration
CTAB	Cetyl Trimethyl Ammonium Bromide
Cs	Cryo-Sensitive
ClO_3^-	Chlorate
CR: NR	Cytochrome-C Nitrate Reductase Activity
Da	Dalton
DEO	1,2,7,8-Diepoxyoctane
DEP	Diethyl Pyro Parbonate
d-ATP	2`- Deoxy-Adenosine 5`- Triphosphate
d-CTP	2`- Deoxy-Cytidine 5`- Triphosphate
d-GTP	2`- Deoxy-Guanosine 5`- Triphosphate
d-TTP	2`- Deoxy-Thymidine 5`- Triphosphate
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DTT	DL-Dithiothreitol
DW	Distilled Water

et al	*Et Alia* (and others)
e.g	*Exempli Gratia* (for example)
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
Fig	Figure
FAD	Flavin Adinine Dinucleotide.Disodium Salt
FMN	Flavin Mono Nucleotide
g	Gram
Glu	Glutamate
GMP	Guanosine Mono Phosphate
GTP	Guanosine Triphosphate
GS	Glutamine Synthetase
h	Hour
i.e	*Id Est* (that is)
KDa	Kilodalton
Kb	Kilobase Pairs
L	Litre
LiCl	Lithium Chloride
LETS	Lithium Chloride Ethylene Diamine Tetra-Acetic Acid Tris- HCl Sodium Dodecyl Sulphate Buffer
LiOA	Lithium Acetate
Meth	Methionine Auxotophic Requirment
μg	Microgram
μl	Microlitre
μCi	MicoCurie
μM	Micromolar
min	Minute

mg	Milligram
ml	Millilitre
mm	Millimeter
mCi	Millicurie
mM	Millimolar
M	Molar
MOPS	3-(N-Morpholino) Propane- Sulphonic Acid
Mo	Molybdenum
ng	Nanogram
nm	Nanometer
nmol	Nanomole
NADPH	b-Nicotinamide Adenine Dinucleotide Phosphate (Reduced Tetrasodium Salt)
NADP-GDH	Nicotinamide Adenine Dinucleotide Phosphate-Glutamate Dehydrogenase
NED	N-1-Naphthyl Ethylene Diamine Dihydrochloride
No	Number
NO_3^-	Nitrate
NO_2^-	Nitrite
NR	Nitrate Reductase
NiR	Nitrite Reductase
NTG	N-methyl-N-nitro-Nitrosoguanidine
°C	Degrees Centigrade
O.D	Optical Density
O/N	Overnight
PABA	Para-Amino Benzoic Acid Auxotophic Requirement

PAGE	Polyacrylamide Gel Electrophoresis
PEG	Polyethylene Glycol
pg	Picogram
pmol	Picomole
PVP	PolyVinylPyrrolidone
KOAC	Potassium Acetate
PMS	Phenazine Methosulfate (N-methyl dibenzopyrazine Methyl Sulfate Salt)
pyro	Pyridoxine auxotrophic requirment
pro	Proline auxotrophic requirment
PCR	Polymerase Chain Reaction
R	Resistant
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions Per Minute
Sec	Second(s)
S	Sensitive
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SDW	Sterile Distilled Water
STC	Sorbitol Tris Calcium Solution
N_3	Sodium Azide
NaOAC	Sodium Acetate
NaOP	Sodium Orthophsphate
TAE	Tris-Acetate Ethelene Diamine Tetraacetic Acid
TE	Tris- Ethylene Diamine Tetraacetic Acid
ts	Temperature-Sensitive

Tris-HCl	Tris(hydroxymethyl) Methlamine
TEMED	N, N, N`, N`-Tetramethyl Ethylene Diamine
u/μl	Unit Per Microlitre
U.A	Uric Acid
U.V	Ultra Violet
Vol	Volume
V/V	Volume To Volume
wA	White Mutation
W/V	Weight To Volume
yA	Yellow Mutation
ZnAc	Zinc Acetate Dihydrate
<	Less Than
>	Greater Than
~	Approximately

SUMMARY.

MOLECULAR STUDIES OF GENES REQUIRED FOR NITRATE ASSIMILATION IN FUNGI AND HIGHER PLANTS.

Ghassan Kana'n.

Nitrate assimilation is an extremely important part of the nitrogen cycle and is carried out by most bacteria, fungi and plants. A relativily short catalytic pathway reduces nitrate to nitrite (via nitrate reductase activity) and then nitrite to ammonium ions (via nitrite reductase activity) which are converted into organic nitrogen by further metabolic pathways. A considerable amount of information is known about the biochemistry, genetics and recently, the molecular biology of these two enzymes. Much less is known about the transport of nitrate and nitrite into cells as well as the synthesis of the molybdenum cofactor needed for nitrate reductase catalytic activity. Research work reported in this thesis focus on these latter two processes in the eukaryotic model organisms, *Aspergillus nidulans* and to a lesser extent *Arabidopsis thaliana*.

Genetic characterisation of 47 *crn* mutants shows that there are three additional genes (i.e. to the original identified *crnA* gene) likely to be involved in nitrate transport. These additional genes are

unlinked to each other or to *crnA*. Although it was shown that the nitrate uptake into cells of these mutants are lower than the wild-type, their exact involvment in nitrate transport requires their molecular cloning. Certain mutations generated in the *crnA* gene have been investigated at the molecular level and the disruptions in the protein determined.

During the genetic studies of *crn* mutants, two other genes were postulated. The first is *chlA*, mutation which results in resistance to chlorate (unlike the wild-type) and caesium (like the wild-type). The second is *cesA* mutation. These latter mutants lead to caesium sensitivity but are chlorate sensitive like the wild-type. These two genes are unlinked to *crnA*, *crnB*, *crnC* and *crnD* genes. The bases of these phenotypes is unclear and need further investigation.

A study of nitrite uptake was undertaken which showed that wild-type *A. nidulans* has an active nitrite transport system. The activity of this system is repressed by ammonium and is nitrate induced. Mutants which are hypersensitive to chlorate taken up much higher levels of nitrite as compared to wild-type.

2,082 *cnx* mutants were isolated and 456 of these were classified as *cnxA*, *cnxB*, *cnxC*, *cnxE*, *cnxF*, *cnxG* and *cnxH* mutants on the basis of phenotypic complementation. No novel *cnx* genes were found. More importantly a number of temperature -conditional mutants were isolated, 10 mutants were found to be temperature-sensitives and 10 cryso-sensitives. Of the

isolated temperature-sensitives 1 located in *cnxA*, 1 in *cnxB*, 2 in *cnxC*, 1 *cnxE*, 2 in *cnxF* and 3 in *cnxH*. Of the crysosensitives 4 in *cnxB*, 3 in *cnxC* and 3 in *cnxF*. These mutants will be particularily useful to relate structure and function when data is forthcoming regarding their protein sequence. Two temperature-sensitive mutants, *cnxH255* and *cnxH261* showed reduced nitrate reductase thermostability which indicates that the *cnxH* product could be associated with the NR protein.

One of the *Aspergillus nidulans* genes required for the synthesis of the molybdenum cofactor was isolated using molecular self-cloning transformation approaches. This gene, *cnxH* , was sequenced at the nucleotide level as well as three mutant alleles (one temperature sensitive and two temperature non-conditional). The results show that the *cnxH* product is the homologue of *Escherichia. coli moaE* whose role is in the synthesis of the molybdenum cofactor specifically to convert the large subunit to active converting factor. Sequence analysis of the two non-conditional mutants indicates that such mutants generated stop codons which provides little or no information about the structure / function relationships. The mutation in the temperature-sensitive mutant lead to a glycine insertion at position +443 and it is postulated that this additional amino acid caused the heat liability of the NR enzyme. Studies of *cnxH* expression show that the *cnxH* is in very low abundance and not regulated at the transcriptional level at least since similar transcript levels were seen in both nitrate and ammonium grown cells-conditions, which making difference for nitrate reductase activity. Finally attempts at isolating *Arabidopsis thaliana cnx* genes failed.

CHAPTER ONE

INTRODUCTION

1.1 The Model Organisms Used In This Study.

Two model organisms which assimilate nitrate were used in this research work. First, the haploid ascomycetous filamentous fungus *Aspergillus nidulans* and second, the diploid higher plant *Arabidopsis thaliana.*

1.1.1 *Aspergillus nidulans.*

The eukaryotic fungus *Aspergillus nidulans* grows on relatively cheap laboratory media requiring only basic growth requirements i.e., an organic carbon and inorganic nitrogen source, water, oxygen, and trace elements. The organism is considered to be relatively easy to handle and safe to deal with, in terms of laboratory research work (Pontecorvo *et. al.*, 1953; and for a recent review see Martenilli, 1994 and refernces therein). Another attraction is that *A. nidulans* possesses a relatively small genome compared to most other eukaryotes, and is distributed over only eight separate chromosomes (Brody and Carbon, 1989) or linkage groups (Clutterbuck, 1994). Moreover, *A. nidulans* possesses a spectrum of genetic systems which can be manipulated including nuclear, organellar (mitochondrial DNA) and extra organellar (plasmids) which provides an opportunity for the study, characterisation and interactions between nuclear,

organellar and the extra organellar genetic systems. Also *A. nidulans* has a variety of cell forms (unicellular, multicellular as well as vegetative asexual and sexual) providing an unique opportunity for the study of eukaryotic developmental and differentiation problems using combined genetical and biochemical approaches. The occurrence of haploid, heterokaryon and diploid cells in a relatively short life cycle, during sexual, asexual and parasexual cycles is very useful (Timberlake and Clutterbuck, 1994). For instance the sexual stage can be used normally for conventional genetic mapping studies, deletion analysis and the examination of inheritance of mutations. The asexual uninucleated spores are perfect for mutagenesis and the fact that the easily seen pigment mutations can be used as markers in genetic crosses. Another most important feature is the occurrence of balanced heterokaryons, in which nuclear migration occurs freely. Finally, the parasexual stage is mainly used for complementation studies, dominance tests, centromere mapping, and the assignment of newly isolated mutations to linkage groups. Additionally, most fungi have the ability to form balanced heterokaryons, through which nuclear migration occurs freely. Heterokaryons can be used for parasexual crosses, which facilitates the solving of many problems such as allelelism questions by complementation tests. Also, this experimental approach helps in the useful analysis of regulatory mutants of gene expression. Fungi in general, are similar to higher eukaryotic organisms in their chromosome structure (nucleosomal organisation, and the presence of histone proteins), mRNA processing,

transcriptional machinary, and therefore are likely to be similar in gene expression mechanisms. However, fungi differ from higher eukaryotes in the lack of abundance of repeated DNA sequences. Moreover, the simplicity of isolating large number of mutants, the development of an efficient transformation protocols and the construction of contiguous physical maps, all help to gather more and more important information. Additionally, the isolation of an array of genes of both scientifical and commercial interest, generates great enthusiasm for the isolation of most genes that lie in this relatively small genome in the near future.

In summary, the main useful features of *A.nidulans*, is that it is a lower eukaryotic organism which is a useful and convenient model system for the study of biological systems. (for a treatise see Martinelli and Kinghorn, 1994 and the various reviews therein, also previously reviewed by Arst, 1981, 1983, 1984, Johnstone, 1985; Mishra, 1985; Hynes, 1986).

1.1.2 *Arabidopsis thaliana.*

The diploid (2n=10, i.e the relatively small genome is distributed over only five pairs of separate chromosomes) higher plant species *Arabidopsis thaliana* belongs to the genus *Arabidopsis* which in turn belongs to the mustard or crucifer family (*cruciferae*). *Arabidopsis thaliana* is widely used for studies of classical and molecular genetics. The utility of *A thaliana* for experiments in molecular biology comes in part from its many advantages including

its relatively small size, short life cycle, screening of large number of mutants, its contribution to the ease of use of this plant in the laboratories and its small nuclear genome. All of these features have reduced the expense and effort required for many types of experiments in molecular genetics. Such correlates as its relative lack of repetitive DNA sequences, reduced copy number of multigene families and reduced size of introns and intergenic spacers (some 60% of the genome consists of single-copy DNA sequences, most of which are genes or associated sequences). These properties facilitate a series of different types of experiments in molecular genetics and allow the cloning of many *Arabidopsis* genes by methods that would be difficult or even impossible if the genome were larger or more typical in its content of repetitive sequences. The structure of individual genes, the structure of chromosomes, the genetic properties, and the overall complement of genes in the genome are typical of those of other flowering plants. Since the above mentioned characters *Arabidopsis thaliana* has been regarded as a useful and therefore popular model organism for investigating a wide range of research topics in plant molecular biology. *Arabidopsis* differs from several of the other model organisms in at least one key respect. *Arabidopsis* is closely related to the species it models. In this respect all angiosperms species are thought to have evolved from a common ancestor, in which they share similar life styles, environmental challenges, and modes of reproduction. An *Arabidopsis* gene may be expected to functionally replace a homologue in many other flowering plants and may be used as a cross-species hybridisation probe to detect

and isolate the corresponding gene. One of the implications of this high degree of similarity between the model and the modelled is that all aspects of *Arabidopsis* biology-development, metabolism, biochemical, environmental, and so are worthy of investigation because of the broad applicability of the gathered information (reviewed in Meyerowits and Somerville, 1994).

1.2 The Nitrate Assimilation Pathway.

1.2.1 Significance.

Nitrogen is an element which is required in greatest amounts by all organisms including eukaryotes such as fungi and higher plants. Nitrogen in the reduced form, is essential for the building of macromolecules required for growth and viability. Inorganic nitrogen is converted to the reduced forms by one of two major pathways. First, nitrogen fixation i.e., the catalytic reduction of the elemental dinitrogen (N_2) to the metabolically usable form ammonium (NH_4^+) by the enzyme nitrogenase (reviewed by Kim and Rees, 1994) and second, nitrate assimilation in which nitrate is converted to again ammonium by a short metabolic pathway (discussed below). Nitrate is the major assimilatory form of nitrogen available for living organisms in the earth's biosphere. In this respect, it has been estimated that more than 10^4 megatons of nitrate are assimilated each year by bacteria, fungi, algae, and higher plants (animals can not assimilate nitrate). Therefore, nitrate assimilation is generally considered to be the major pathway being 100 fold greater

than the total amount of the alternative pathway of nitrogen fixation, which is carried out by leguminous plants in association with symbiotic bacteria. Therefore, it is a very important pathway for nitrogen reduction and is present in most prokaryotic and eukaryotic organisms including bacteria, algae, fungi, and higher plants (For reviews see Cove, 1979; Unkles, 1989; Kim and Rees, 1994; Crawford, 1995 and refernces therein). Unlike in eukaryotes, bacterial nitrate reduction can serve two main purposes, nitrate can be reduced to ammonium and used as a nitrogen source for the synthesis of nitrogenous cellular components. Alternatively, nitrate can be reduced to nitrite, ammonium, nitrous oxide, or dinitrogen for anaerobic respiration, which generates energy for cell growth.

Nitrate assimilation is of considerable importance for agriculture, the environment and human health. In agriculture, nitrate based fertilisers are widely used in order to increase the crop yield. However the usage of huge amounts of nitrogen based of fertilisers has several inherent disadvantages including: (1) the cost, (2) production of environmentally unfriendly nitrous gases. (3) compounds that damage animal and human health. In the latter regard, health problems may result from the leaching of N-nitroso compound into drinking water. Later these compounds may be find the way into animal and the human gut (Forman and Shunker, 1989). One research hope is that the dissection and elucidation of the nitrate assimilation system will hold long term promise for solving these problems. In agriculture the ambition is to increase crop yields and

reduce the amount of nitrate fertilisers required. In addition, to reduce human and animal health risks from carcinogenic N-nitroso compounds derived from nitrate. Further and from an ecological point of view, another objective is to improve the utilisation of nitrogen by mycorrhizal fungi in nitrogen deficient soils (reviewed in Martin and Botton, 1993).

1.2.2 The Nitrate And Ammonium Assimilation Pathways And The Purine Degradation Pathway.

Nitrate assimilation pathway involves three sequential steps, where nitrate uptake from the external environment, and its release into cells by nitrate transporter(s) is most likely the first step in this assimilation pathway (Figure 1.1). Two further enzymic steps then occur: reduction of nitrate to nitrite by the activity of nitrate reductase (NR) (NADPH: nitrate oxidoreductase, E.C.1.6.6.3), then the conversion of nitrite to ammonium by nitrite reductase (NiR) (NADPH: nitrite oxidoreductase E.C.1.6.6.4) (reviewed in Cove, 1979; Scazzocchio and Arst, 1981; 1983; Kinghorn and Unkles, 1994 and references therein).

In *Aspergillus nidulans* genetical and biochemical studies have indicated that the *crnA* gene encodes a nitrate transporter (Brownlee and Arst, 1983; Unkles *et. al.*, 1991). The evidence which supports *crnA* being a structural gene for nitrate uptake came from the following findings: First, genetical studies have revealed that *crnA* mutants showed resistance to chlorate with wild-type growth on

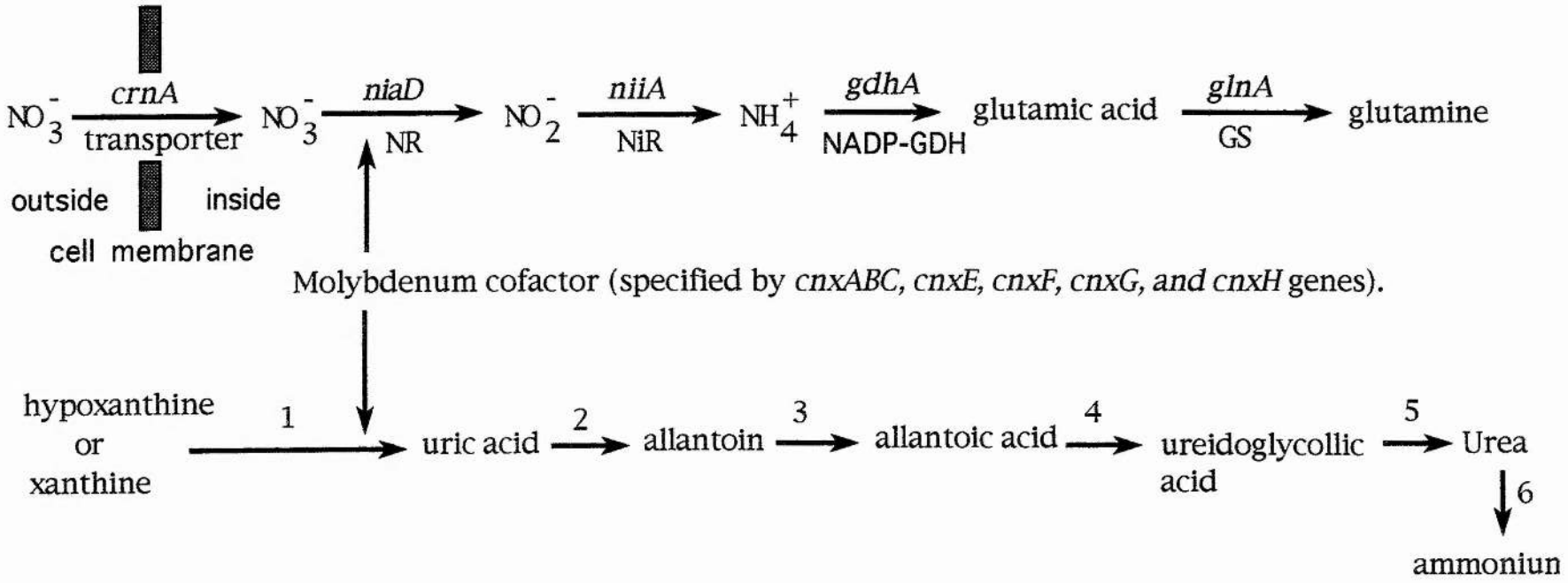
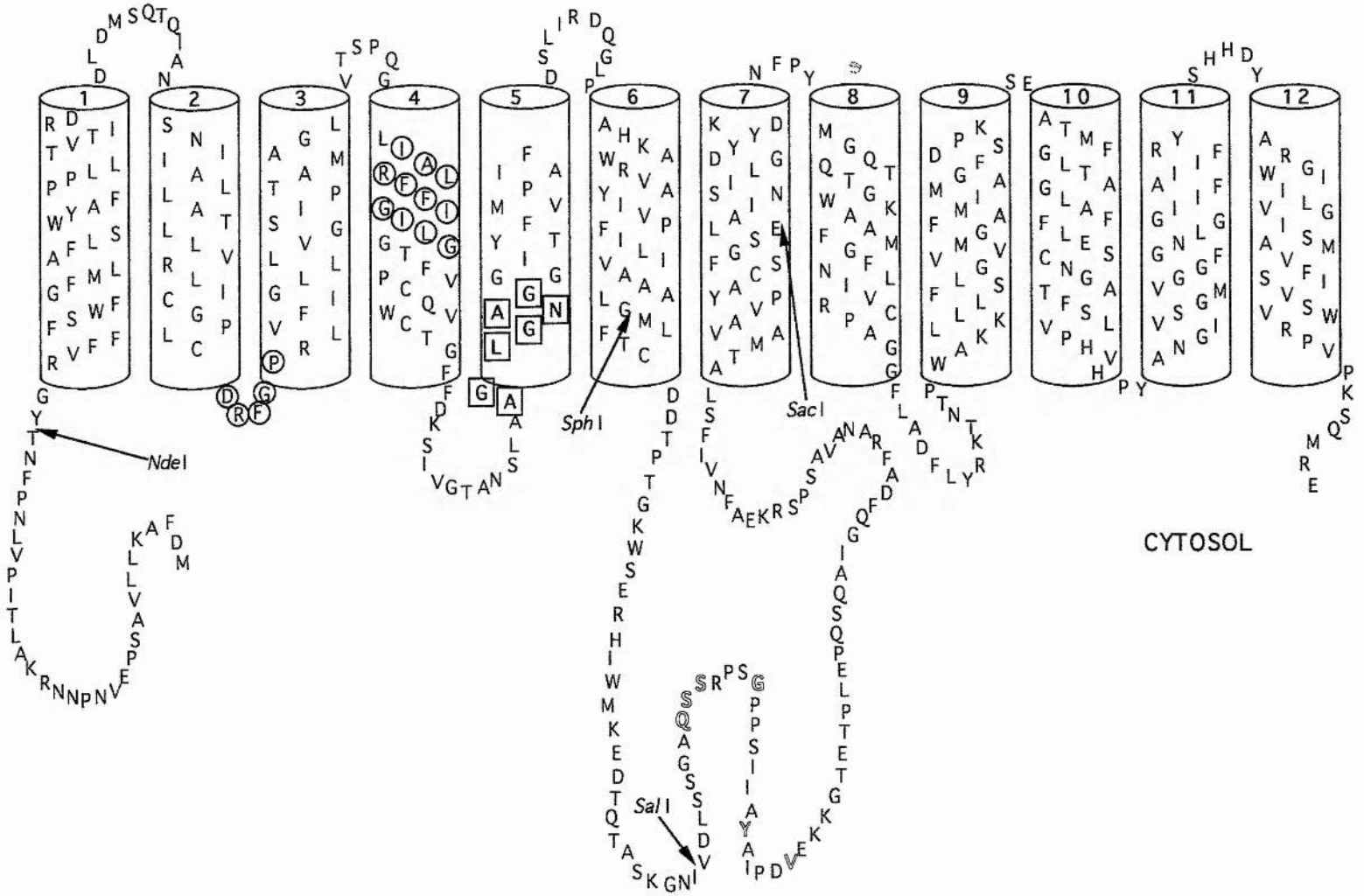


Figure 1.1. Nitrate Assimilation Pathway And Functionally Related Pathway. Genes and enzymes involved in nitrate and ammonium assimilation, and purine degradation enzymes, are shown above. Other genes and enzymes including control genes involved in the regulation of these pathways: *nirA* gene, positive-acting gene for nitrate induction, and *areA* gene, positive-acting gene for nitrogen metabolite repression. In purine degradation pathway 1: xanthine dehydrogenase induced by xanthine; 2: urate oxidase induced by uric acid and xanthine; 3: allantoinase; 4: allantoicase; 5: ureidoglycollase; 6: urease. Hypoxanthine is not an inducer for enzyme 1 (xanthine dehydrogenase), but it acts through its conversion to uric acid, thus enzymes 1 and 2 share both induction and regulation (Pateman, *et. al.*, 1964; Darlington *et. al.*, 1965, reviewed in Cove, 1979; Unkles *et. al.*, 1989; Kinghorn and Unkles, 1994).

Figure 1.2. Secondary Structure Model For CRNA Protein.

The *CRNA* protein is generally considered to be a nitrate transporter with a polypeptide of 52 KDa. It's hydropathy profile shows 12 hydrophobic membrane-spanning helices. The *CRNA* transporter belongs to the major facilitator super family (MFS) of transporters, which share a common structure of 12 transmembrane ∝- helices. The proposed orientation of *CRNA* with the N-and C-termini on the cytosolic side of the membrane conform to the 'positive inside rule' which predicts the majority of positively charged amino acid residues to be present inside the cell. MFS transporters have two consensus motifs observed between transmembrane domains 2 and 3 and 1-X2-R-X3-G-X3-G at the beginning of and extending into transmembrane domain 4 (circles), as well as a motif known as the 'binding-protein-dependent transporter system inner membrane component signature', (E,Q)-(S,T,A)2-X3-G-X6-(L,I,V,M,Y,F,A)-X4-(F,I,L,V)-(PQ) in the large cytoplasmic loop between transmembrane segments 6 and 7 (letters in outline). The central loop, which is unusually long (95 amino acid residues) in *crnA*, has been shown to be functionally important in other MFS proteins such as the rat glucose transporter,*Glut4*. Comparisons between amino acid sequences of fungal, algal, and higher plants nitrate transporters revealed a conserved motif at the beginning of transmembrane domain 5 (cytoplasmic side), A-G-(W,L)-G-N-M-G (squares), not present in other MFS proteins, which may be a signature for nitrate/nitrite transporters. Topological models for the algal, fungal, and higher plant proteins differ with regard to the size of the central loop between transmembrane domain 6 and 7. The *CRNA* central loop which is localised to the cytosolic side of the membrane is 95 amino acid residues long, whilst the algal, and higher plant ones are smaller 34, and 36 amino acids, respectively. IN contrast, the C-terminal domain which is only 7 residues in *CRNA* is much longer-92 residues in the algal *NAR3*, 73 in *NAR4*, and 67 in the plant *BCH1* and *BCH2*. It has been suggested that in these proteins this domain may perform some of the functions which are carried out by the central loop of *CRNA* (Trueman *et. al.*, 1996).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SphI
SacI
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nitrate as sole nitrogen source (Tomsett and Cove, 1979; Brownlee and Arst, 1983). Second from biochemical evidence, *crnA* mutants had reduced the uptake of nitrate (Brownlee and Arst, 1983). Third, molecular analysis (Unkles *et. al.*, 1991) revealed that *crnA* gene has specified a polypeptide of 483 amino acids (molecular weight of 52 KDa) with 12 membrane spanning segments, which may form the channel for nitrate transport. Finally, classical genetic studies showed that the *crnA* gene maps close to the other two structural genes for nitrate assimilation namely *niiA* and *niaD* (Tomsett and Cove, 1979; Reviewed in Cove, 1979).

Genetic studies have shown that *niaD* gene is the structural gene for the NADPH NR apoprotein, that catalysis the reduction of nitrate to nitrite. Mutants within the *niaD* gene results in chlorate resistance and the inability to utilise nitrate as the sole nitrogen source, but grow normally with other nitrogen sources (reviewed by Cove, 1979). Recent biochemical and molecular studies (Johnstone *et. al.*, 1990) revealed that *niaD* gene encodes a protein of 873 amino acids with a subunit size of 95 KDa. The results obtained by Johnstone *et. al.*, (1990) along with others (Minagawa and Yoshimoto, 1982) implicated that the *A. nidulans* NR is a homodimeric structure with a 180 KDa molecular weight and a subunit size of 91 to 95 KDa (the *niaD* gene product) (Tomsett, 1991).

Recently *niiA* gene the structural gene for NiR has been sequenced, and the protein was found to consist of a polypeptide of

1104 amino acids with a molecular weight of 126 KDa (Johnstone *et. al.*, 1990). Mutations in the *niiA* gene lead to the abolition of NiR activity which is required for the conversion of nitrite to ammonium in the nitrate assimilation pathway. Additionally, *niiA* mutants are unable to utilise nitrate and nitrite as sole nitrogen sources. In contrast, to *crnA* and *niaD* mutants, *niiA* mutants show sensitivity to chlorate. Classical genetic studies revealed that the three structural genes for nitrate assimilation pathway (*crnA-niiA-niaD*) are tightly linked in a cluster in the order *crnA-niiA-niaD* on linkage group VIII of the *Aspergillus* genetic map Figure 1.3 (Tomsett and Cove, 1979; Arst *et. al.*, 1979). The expression of *crnA*, *niiA*, and *niaD* genes are subject to induction by nitrate (mediated by the product of the *nirA* regulatory gene product) and to ammonium or nitrogen metabolite repression (by the *areA* regulatory gene product) in addition, to autoregulation by *niaD* itself (see section 1.7.5 regulatory genes for nitrate assimilation) (reviewed by Cove, 1979; Scazzocchio and Arst, 1989, 1994). It is interesting that in its near relative, *N. crassa* , the equivalent genes of *niiA* and *niaD* (i.e. *nit-1* and *nit-3* respectivily) are unlinked to each other and located on different chromosomes (Tomsett and Garrett, 1980).

Ammonium formed from this pathway is assimilated into glutamate by the catalytic reaction of NADP-linked glutamate dehydrogenase (NADP-GDH), which is encoded by the *gdhA* gene which maps in linkage group III (Arst and MacDonald, 1973; Kinghorn and Pateman, 1973). The final step in ammonium

assimilation is the conversion of glutamate into glutamine by glutamine synthase (GS), which is encoded by *glnA* gene and maps in linkage group II (MacDonald, 1982; Cornwell and MacDonald, 1984). Of peripheral, but noteworthy interest, a further glutamate dehydrogenase (NAD-GDH) encoded by the *A nidulans gdhB* gene which maps in linkage group IV (Arst *et. al.*, 1975; Kinghorn and Pateman, 1976) and it is unlinked to *gdhA*. In fact these genes locate on different chromosomes. Previous studies suggested that NAD-GDH might have a reverse role to *gdhA* i.e converting glutamate to ammonium where ammonium is an short supply. No *gdhB* or *glnA* DNA clones have been reported recently to investigate the expression of *gdhB* (reviewed in Kinghorn and Unkles, 1994).

The first steps of purine degradation pathway in *A. nidulans* involve two purine hydroxylases (formerly named xanthine dehydrogenase I and II) namely purine hydroxylases I and II which were described some time ago by Scazzocchio *et. al.*, (1973) in *A.nidulans*.. Both hydroxylases were found to be different with regard to their substrate affinity and their regulation of expression. Purine Hydroxylase I was found to utilise hypoxanthine as a substrate, its activity induced by uric acid whilst repressed by ammonium. Loss of activity leads to the inability to use hypoxanthine, but not uric acid, as sole the nitrogen source. On the other hand purine hydroxylase II is an nicotinate inducible enzyme, and is able to utilise both hypoxanthine and nicotinate as sole nitrogen sources and loss of activity leads to inability to use nicotinate (but not 6-

hydroxynocotinate) and hypoxanthine. Both ammonium and nitrate act as co-repressors of this enzyme (Darlington *et. al.*, 1965; Darlington and Scazzocchio, 1968, Scazzocchio, 1973). Mutation in the *cnx* genes leads to loss of activity of both purine hydroxylase I and II, in addition to the loss of nitrate reductase activity. Three temperature-sensitive *cnxH* mutants, isolated by MacDonald and Cove (1974) showed growth with nitrate at 25°C, but showed the mutant phenotype with hypoxanthine at the same temperature. In addition, the three temperature-sensitive *cnxH* mutants, exhibited a heat labile nitrate reductase enzyme but not purine hydroxylase I and II at 25°C. Such results suggested that *cnxH* gene might code for a structural component required for the activity of all three enzymes (MacDonald and Cove, 1974; Scazzocchio, 1974; and reviewed in Cove, 1979). Both purine hydroxylase I and II were found to be able to hydroxylate hypoxanthine. The gene *hxA* (the structural gene for purine hydroxylase I) lies on chromosome V has been cloned and sequenced. The protein is a 304 KDa homodimer with each subunit contain two Fe-S centres, FAD and Mo. The gene *hxnS* which encodes a structural component of purine hydroxylase II, lies on chromosome VI (Lewis *et al.*, 1978). Purine hydroxylase II from *A. nidulans* has been purified, and found to have a molecular weight of 300 KDa, consisting of two subunits of molecular weight of 153 KDa. This enzyme contains 4 (2 Fe-2 S) Iron-sulphur centres, 2 FAD, 2 Mo atoms per molecule, and has the ability to oxidise hypoxanthine and nicotinate but not xanthine (Mehra *et. al.*, 1984).

1.3 Nitrate And Nitrite Transport.

1.3.1 Biochemistry.

Whilst much detailed information is available regarding the nitrate assimilatory enzymatic steps including their structure, function and regulation, much less is known about the nitrate and nitrite uptake systems in both prokaryotes and eukaryotes. Uptake systems (particularly nitrate) must, *a priori*, be fairly responsive, robust and versatile since probably all organisms need effectively to take up considerable amounts of nitrate to satisfy their nitrogen requirments for growth. in for example soil environments, in which the concentration of such essential nitrogen source may vary greatly. Biochemical research work with *A. nidulans* provided evidence for a distinct nitrate-inducible nitrate uptake system with a Km value of around 200 mM (Brownlee and Arst, 1983). However, the *Aspergillus* nitrate uptake system was found to be different from the formerly nitrate-inducible nitrate uptake systems (with Km's around 250 μM) in other related fungi, such as the breadmould *Neurospora crassa* (Schloemer and Garrett, 1974; *Quesada et. al.*, 1994) or the industrial antibiotic producer *Penicillium chrysogenum* (Goldsmith *et. al.*, 1973). The *Aspergillus* nitrate uptake system requires a functional nitrate reductase activity in order to be active. In addition, transport studies on a strain carrying the *crnA1* mutation resulted in reduced net nitrate uptake up to 4-folds in conidia and young mycelial cells (8 h). In contrast, this mutation seemed to have no effect on older cells (16 h) with regard to nitrate transaport (Brownlee and

Arst, 1983). These findings might suggest that, *crnA* gene encodes a differentially regulated component expressed in spores and young mycelia but, not in older cells. Their work also indicated that, more than one nitrate uptake system was present, one system for conidia and young cells, whilst the other in older cells (Brownlee and Arst, 1983; reviewed by Cove, 1979; Pombeiro *et. al.*, 1983; Crawford, 1995).

Research studies were carried out in order to determine the effect of different inducers or inhibitors on the nitrate and nitrite transport systems. Brownlee and Arst (1983) in *A. nidulans* reported that, both nitrate and nitrite induces the uptake of nitrate in the wild-type mycelium, where the degree of induction by nitrite was lesser than that of nitrate, this variability in induction was related to the slight toxicity of nitrite, in addition to its reactivity precluding its use. Additionally, they showed that chlorate the toxic analogue of nitrate, strongly and rapidly inhibits the net nitrate uptake in both young and older cells of wild-type and *crnA1* strains in *A. nidulans*.

In *N. crassa* , Schloemer and Garrett (1974) reported that nitrite as an inducer for a nitrate uptake system(s). It affects negatively nitrate uptake rates also the mechanism of nitrite inhibition was suggested to be non-competitive. These *N. crassa* results agreed broadly with those previously obtained in in the duckweed *Spirodela* (Ferguson and Ballard, 1969), and Tobacco *Nicotiana* cells (Heimer and Filner, 1971).

Using *Sirodela. oligorrhiza* Ferguson and Ballard, (1969) demonstrated that when nitrate-grown *Sirodela* cells were supplied with both nitrate and ammonium at the same time, ammonium was utilised first. The authers showed that the uptake could occur in the absence as well as in the presence of a functional (NR) activity, i.e without noticeable sudden changes in the enzyme level. These findings lead to the suggestion that ammonium might prevent nitrate uptake by a mechanism(s) other than repressing enzyme activity *vis-a vis* in a non-competitive inhibitory manner principally.

Galvan and his colleagues studied and characterised nitrite and nitrate transport in nitrate transport-deficient mutants of the alga *Chlamydomonas reinhardtii*, as well as the genetic transformants carrying either *nar 2* and *Nrt 2;1* or *nar 2* and *Nrt 2;2* genes (Galvan *et. al.*, 1996) as discussed before. The results obtained indicated that nitrate transport- deficient mutants grow on nitrite and show nitrite uptake activity at lower nitrite concentrations (i.e < 25 μM) but not nitrate. This led to the conclusion that the uptake system present in such strains is nitrite specific. In addition, integration of the *nar 2* and *Nrt 2;1* genes into the nitrate transport-deficient mutants genome led to increase in the nitrite uptake rates of 4 to 5-fold. Such data would suggest that an integrated system mediates the entrance of nitrite into the cell even more efficiently than the specific nitrite transport system in the nitrate transport-deficient strains i.e., it is a bispecific system (Galvan *et. al.*, 1996). However, transformation and integration of *nar 2* and *Nrt 2;2* genes into the nitrate transport-

deficient mutants genome, resulted in similar nitrite uptake rates to those in the parental strains (i.e nitrate transport-deficient strains). This indicated that integration resulted in a system which is specific for transporting nitrate but does not transport nitrite efficiently (Galvan *et. al.*, 1996).

Regarding the effect of different inhibitors on nitrite uptake, Cordoba *et. al.*, (1986) studied the kinetic characteristics of nitrite uptake and its inhibition by nitrate in the wild-type strain of Chlamydomonas *reinhardtii*. The results demonstrated that nitrate can be considered to be as partially competitive inhibitor of nitrite uptake in which inhibition is independent of the existence of functional (NR). These results appeared to fit with the presence of different transport system for nitrite and nitrate at least in *Chlamydomonas reinhardtii*. Competition by nitrate and its toxic analogue chlorate (see section 1.7.1 on chlorate toxicity) have been studied in *C. reinhardtii* by Galvan *et. al.*, (1996). The latter researchers found that neither nitrate at a concentration of 50 μM nor chlorate at a concentration of 1.5 mM had an inhibitory effect on the uptake of nitrite by the nitrate specific system (*nar 2 / Nrt 2;2*). In contrast, both ions (i.e nitrate and chlorate) were found to inhibit significantly nitrite uptake in the bispecific system (*nar 2 / Nrt 2;1*), which transport both nitrate and nitrite efficiently (Galvan *et. al.*, 1996). However, a decade age, Cordoba *et. al.*, (1986) studied the effect of different nitrate concentrations on the uptake of nitrite by the nitrite specific transporter (i.e independent on the *nar 2* gene). They

reported that nitrate up to a 100 μM did not have any significant effect on the uptake of nitrite. Very recent research work carried out by Galvan *et. al* (1996) showed that different nitrate concentrations up to 75 μM had no effect on the uptake of nitrite by either the nitrite specific (i.e nitrate transport-deficient system) or the nitrate specific systems (*nar 2 / Nrt 2;2*). In contrast, nitrate at a concentration of up to 75 μM strongly inhibited the uptake of nitrite by the bispecific system (*nar 2 / Nrt 2;1*).

1.3.2 Genetics.

Genetic analysis of nitrate assimilation in *A. nidulans* revealed that *crnA* gene is one of three tightly linked genes in a cluster located in chromosome VIII, the gene order being *crnA-niiA-niaD*. The *crnA* gene (*crn* = **c**hlorate **r**esistant **n**itrate utilising) of *A. nidulans* was first revealed by mutations therein, which were selected on the basis of chlorate resistance (Tomsett and Cove, 1979; reviewed by Cove, 1979). A number of genes may affect nitrate and/or nitrite uptake and these genes are discussed below.

crn mutants can be easily and simply isolated, screened and distinguished from other nitrate assimilation mutants such as *niaD* mutants by their ability to grow as the wild-type at a range of concentrations on nitrate or nitrite as the sole nitrogen sources (Brownlee and Arst, 1983; and reviewed in Cove, 1979).

No nitrite uptake gene has been described in *A. nidulans*. Mutations in four unlinked genes have been reported to be phenotypically hypersensitive to nitrite. First, the *nihA* mutation maps in linkage group I (Pombeiro *et al.*, 1983), results in sensitivity to higher levels of nitrite (≥10 mM), but on normal concentrations it grows as wild-type. This lead to the suggestion that such mutation makes the strain more efficient in nitrite uptake, or more efficient in the efflux of nitrite back into the medium. The second, nitrite hypersensitive mutation is *niiC628* which maps in linkage group I (reviewed in Cove, 1979). This mutant grows on both nitrate and nitrite (10 mM) with a nitrogen-starved morphology, has the wild-type levels of both (NR and NiR) enzymes activity. As before, this suggests that there is an altered permease function in the mutants resulting in either increase, influx, or decreased efflux. (Cove, 1979). The third gene is *meaB6*, which maps in linkage group IV *meaB* mutants have both NR and NiR activities and are also hypersensitive to nitrite (≥10 mM). In contrast to the other hypersensitive mutants, *meaB* mutants show resistance to methylammonium (100 mM) when nitrate (10 mM) is the sole nitrogen source (Arst and Cove, 1973; Arst and Page, 1973). Very recently, on the basis of the reversal of nitrite hypersensitivity (as the selection system), Polley and Caddick (1996) cloned and later sequenced the *meaB* gene. This gene was found to encode a novel protein product of 418 amino acids, which it is suggeted, by the authors to be a new transcriptional regulatory protein. Additionally, sequence analysis of the mutant *meaB6* has indicated that it is a single base pair deletion, which terminates the

translation at residue 233 which in turn, resulted in frame shift mutation leading to truncation of the *meaB* product. These findings indicated that the situation regarding the status of nitrite efflux and nitrogen metabolite repression in *meaB* locus is still unclear (Arst and Cove, 1969; reviewed in Cove, 1979). The fourth nitrite hypersensitive mutation (10 mM) is the *tamA105* which maps in linkage group VI. The *tamA105* mutant was selected for resistance to thiourea (i.e a toxic analog of urea), aspartate hydroxylate (toxic analog of asparagine), and chlorate (toxic analog of nitrate), in addition to its resistance to methylammonium (toxic analog of ammonium) (Kinghorn and Pateman, 1975). Very recently, the *tamA* gene was cloned and sequenced (Davis *et. al.*, 1996). From the inferred amino acid sequence analysis it is pridicted that the *tamA* gene encodes a putative control protein with a putative zn(II)2 cys-6 cluster DNA binding domain. Surprisingly the zinc cluster DNA binding domain has been found to be not required for function. In contrast, a small internal in-frame deletion within the *tamA* gene has been found to abolish *tamA* function. Therefore, it is likely that this deleted region and possibly other conserved region are the important functional domains of this protein (Davis *et. al.*, 1996). The function of the *tamA* gene is still unknown, but it could be involved in nitrite uptake (Kinghorn and Pateman, 1975).

1.3.3 Molecular Biology.

The *crnA* of *Aspergillus. nidulans* has been found to encode a nitrate transporter, a membrane protein with 12 membrane-spanning

segments (Unkles *et. al.*, 1991). From this molecular data, it was concluded that the *crnA* gene of *A. nidulans* encodes a high affinity nitrate transporter, and this conclusion arises from different lines of evidence. First, the *crnA* protein contains twelve hydrophobic membrane-spanning helices (Figure 1.2, secondary structure prediction using TMAP) (Unkles *et. al.*, 1991; Trueman *et. al.*, 1996). Second, Northern blot results indicated that *crnA* expression is induced by nitrate and repressed by ammonium, which is similar to other genes in the pathway such as *niaD* (NR) and *niiA* (NiR) confirming its association with the nitrate assimilation pathway. Third, reduced net nitrate uptake is observed in the *crnA1* mutant relative to the wild-type strain (Brownlee and Arst, 1983; Unkles *et. al.*, 1991).

Since the publication of *crnA* sequence, other eukaryotic high affinity transporters have been identified and characterised at the DNA and protein level. In the green alga *Chlamydomonas reinhardtii* , two nitrate transport protein carriers have been identified. The algal transport proteins were found to be encoded by *Nrt 2;1* and *Nrt 2;2*, genes (formerly named *nar 3* and *nar 4*, respectively) with the requirement of a functional *nar 2* gene as discussed above. The function of the *nar 2* gene is not clear yet but, it has been suggested that *nar 2* product is either a structural or regulatory protein required for the function of both high affinity transport genes (*Nrt 2;1* and *Nrt 2;2*) (Quesada *et. al.*, 1994). The *C. reihardtii* transport proteins (*Nrt 2;1* and *Nrt 2;2*) showed high identity at the

amino acid level with each other (almost 80% identical) and with the *Aspergillus CRNA* nitrate transport protein (about 31% identity) (Fernandes *et. al.*, 1989; Trueman *et. al.*, 1996; Unkles *et. al.*, 1991; Quesada *et. al.*, 1994; reviewed in Crawford, 1995). The algal (*Nrt 2;1* and *Nrt 2;2*) and the fungal (*CRNA*) nitrate transport proteins were showning to be hydrophobic proteins having 12 hydrophobic membrane-spaning segments, which would form the channel for nitrate transport (Johnstone *et. al.*, 1990; Unkles *et. al.*, 1991; Quesada *et. al.*, 1994). Very recently, in *C. reinhardtii* nitrate transport deficient mutants and transformed strains of *C. reinhardtii* carrying different sets of *nar 2* genes have been molecularly and functionally characterised. The nitrate transport mutants have been complemented by transformation for growth on media containing 2 mM nitrate with either a plasmid carrying *nar 2* and *Nrt 2;1* genes, or a plasmid carrying *nar 2* and *Nrt 2;2* genes respectively. The obtained results led to the conclusion that three different nitrate/nitrite transport systems may operate in the green alga *C. reinhardtii.* As discussed before the first uptake system is a nitrite specific transporter, which is independent of the *nar 2* gene product, and two additional nitrate transport systems. The second is specific for nitrate and this encoded by *nar* 2 */Nrt 2;2* genes. The third system transports both anions efficiently and is encoded by the *nar 2 /Nrt 2;1* genes (Galvan *et. al.*, 1996).

In higher plant cells, it is generally believed from physiological experiments that at least two nitrate transport systems are involved. The first is a high affinity system which is inducible by nitrate, the other is a low affinity system expressed constitutively (Hole *et. al.*, 1990; Glass *et. al.*, 1992). A putative nitrate transporter cDNA clone has been isolated from the higher plant *Arabidopsis thaliana* (Tsay *et. al.*, 1993). Perhaps surprisingly, this clone encodes a protein which does not show amino acid similarty with any other algal or fungal high affinity nitrate transporter (Tsay *et. al.*, 1993; Quesada *et. al.*, 1994). Very recently a gene from the higher plant *Hordium vulgare* (barley) has been cloned using primers based on the *A. nidulans crnA* sequence (Trueman *et. al.*, 1996). This barley clone has been also found to encode a protein with 12 putative membrane-spanning segments, and it showed identity at the amino acid level with the *Aspergillus CRNA* protein (identity to 32%) and with the algal *NRT 2;1* protein (identity to 50%) (Trueman *et. al.*, 1996). Although no functional data has been reported for the barley protein, its significant similarity to other CRNA transporter. The plant is likely by extension to encode a high affinity nitrate transporter. Since the nitrate transport systems in higher plants appear to be more complex than lower organisms, the existence of specific nitrite transporter(s) is still uncertain (Hoff *et. al.*, 1995). Not unexpectedly, no *S. cerevisiae* nitrate transporter(s) homologs have been identified from the yeast genome data base (S.E Unkles, unpublished). That yeast does not utilise nitrate also supports the notion that the CRNA protein from nitrate utilising *A. nidulans* , is indeed a a nitrate transporter.

1.4 Nitrate Reductase And Nitrite Reductase.

1.4.1 Biochemistry.

It has been reported that three closely related forms of nitrate reductase are present in algae, fungi, and plants (Wray and Kinghorn, 1989 and references therein). First, there is NADH NR (E.C.1.6.6.1) which is the most common form observed in most higher plants and algae. Second, certain plants possess NADPH NR (E.C.1.6.6.2). In fungi, NADPH NR (E.C.1.6.6.3) is only present. NR is found to be a multi-redox enzyme having 3 functional domains associated with 3 prosthetic groups, FAD, heme-Fe, and molybdopterin (reviewed by Dunn-Coleman *et. al.*, 1984 and references therein). The 3 functional domains of NR bind its 3 cofactors in order to form the active sites of the enzyme. It has been shown that NR physiological activity (*in vivo*) involves the transfer of 2 electrons from NADP(H), through FAD, heme, and Mo-Co, to nitrate (Cooley and Tomsett, 1985; Johnstone *et. al.*, 1990; Garde *et. al.*, 1995; reviewed by Campbell and Kinghorn, 1990 and references therein).

The *A. nidulans* wild-type NR (and related fungal species such as *N. crassa*) has been shown to possess an NR associated cytochrome-C reductase activity, basically a further NR related partial activity (Cove and Coddington, 1965). Pateman and co-workers (1964) demonstrated that such cytochrome-C reductase (NADPH: cytochrome-C oxidoreductase, E.C.1.6.2.3) activity is retained in *cnx* and *niaD* mutants. This partial activity was found

even in cells grown in the absence of nitrate. Two protein species showing cytochrome-C reductase activity were detected in the wild-type mycelium, grown in the presence of nitrate (Table 1.1). One species with a sedimentation coefficient of 7.6s (identical to that of NR) which is induced by nitrate and repressed by ammonium, the other (13.7s)which was also detectable in *cnx* mutants of 13.7s. Since this species was found to be present in the wild-type as well as in (all) *cnx* mutants (which lack NR activity), and because it is neither induced by nitrate nor repressed by ammonium, it was suggested that 13.7s molecule is not involved in nitrate reduction. Instead, it might act in association with some other enzyme such as sulphite reductase and therefore ignored in this work. The *cnx* mutants can be divided into two groups: The first, with nitrate inducible cytochrome-C reductase activity, where the 7.6s species can be detected at the same level or even a higher level than that of the wild-type e.g. *cnxB13*, *cnxF9*, *cnxG2*, and *cnxH1* mutants. The second group consist of, constitutive cytochrome-C reductase mutants that can produce a new species (absent in the wild-type) of sedimentation coeffiecent 4.5s e.g. *cnxA5*, *cnxB11*, *cnxF2*, *cnxG4* and *cnxH3*. Both *cnxE* constitutive and inducible mutants (unlike other *cnx* mutants) produce only the 7.6s species (not the 4.5s species). Some strains of *A. nidulans* such as *cnxB13*, *cnxF9*, *cnxG2*, and *cnxH1* produced 7.6s, and 4.5s when grown on urea and nitrate i.e. induced. This lead to the suggestion that the 4.5s which was present only in mutants lacking NR activity, might be a subunit of native 7.6s NR. It was proposed that the 4.5s is specified by the *niaD* gene the NR structural gene, and in the

Table 1.1. Sedimentation Coefficients Of NADPH Cytochrome-C Reductase From Wild-Type And Selected Mutant Strains Of *A. nidulans* (MacDonald *et. al.*, 1974).

The 13.7s subunit was found to be present in the wild-type and all *cnx* mutants examined. This subunit unlike NR itself is neither induced by nitrate nor repressed by ammonium. Therefore MacDonald *et. al.*, (1974) suggeste that it could be involved in an alternative activity of other and unrelated enzymes (such as sulphite reductase). The 4.5s species may be is a subunit or breakdown product of the 7.6s NR protein specified by *niaD* gene. The 4.5s species has been found in all *cnx* mutants constitutive for cytochrome-C reductase but, not in either *cnxE* or wild-type strains. Mutants that produce 4.5s species (lacking Mo-Co) are unable to aggregate the NR enzyme subunits (ie. 4.5s can't aggregate to form 7.6s molecules). Some *niaD* mutants have no 4.5s or 7.6s cytochrome-C reductase. The 7.6s present in all inducible *cnx* mutants except *cnxE* mutants. Both constitutive and inducible *cnxE* mutants produce only the 7.6s at levels greater than the wild-type levels. The 7.6s was considered to be a dimer ot two 4.5s *niaD* products, and one or more co-factor necessary for the 7.6s enzyme to show NR activity as well as for the dimerisation of the 4.5s *niaD* gene product to show NR activity when this molybdenum co-factor remains bound to the dimer. If the molybdenum co-factor dissociate it will leave the 7.6s subunit having cytochrome-C reductase activity but not NR activity.

Mutant	Induced(I) or Constitutive Mutation (C)	Sedimentation Coefficient (Cells Grown With 5 mM Urea As the Sole Nitrogen Source (i.e.Uninduced Conditions)	Sedimentation Coefficient (Cells Grown With 5 mM Urea and 10 mM Nitrate (i.e. Induced Conditions)
biA1	I		7.6s
yA2 wA3 cnxA4	I		4.5s
biA1 cnxA5	C	4.5s	4.5s
biA1 cnxB13	I		7.6s and 4.5s
biA1 cnxB11	C	4.3s	4.4s
biA1 cnxE14	I		7.6s
biA1 cnxE13	C	7.8s	7.8s
yA2 wA3 cnxE3	C	7.7s	7.7s
biA1 cnxF9	I		7.6s and 4.5s
yA2 wA3 cnxF2	C	4.5s	4.5s

yA2 wA3 cnxE3	C	7.7s	7.7s
biA1 cnxF9	I		7.6s and 4.5s
yA2 wA3 cnxF2	C	4.5s	4.5s
yA2 wA3 cnxG2	I		7.6s and 4.5s
biA1 cnxG4	C	4.4s	4.4s
yA2 wA3 cnxH1	I		7.6s and 4.5s
biA1 cnxH3	C	4.5s	4.4s
yA2 wA3 niaD8	C		
yA2 wA3 niaD 10	C		
yA2 wA3 niaD 13	C		
biA1 niaD 17	C	4.5s	4.5s

presence of the molybdenum cofactor, the 4.5s aggregates to form the 7.6s product. Only the 7.6s species complexed with the molybdenum cofactor possesses NR activity. When dissociated into 4.5s subunits only the alternative activity (cytochrome-C reductase activity) is observed. Consequently this lead to the conclusion that mutants, which produce the 4.5s species were unable to aggregates the enzyme subunits into native NR, i.e., the 4.5s is unable aggregate to form the 7.6s molecules (MacDonald *et. al.*, 1974; reviewed in Cove, 1979).

In summary the *niaD* gene seems to specifies a 4.5s protein with cytochrome-C reductase activity only. The 7.6s molecule, a dimer of two 4.5s (*niaD*) products and one or more molybdenum cofactor molecules (*cnx* genes) has both NR and cytochrome-C activities. The molybdenum cofactor, therefore, is necessary for the dimerisation of the 4.5s products in order to provide enzyme activity. In other words, when the molybdenum cofactor is in the form of a complex with the 4.5s dimer, it shows NR activity but, when dissociated it only shows the alternative cytochrome-C NR activity.

The most widely used assay method for the presence of Mo-Co, is the *in vitro* complementation of the nitrate reductase subunits in *nit-1* extracts of *N. crassa* mutant (deficient in the synthesis of precursor Z in Mo-Co biosynthesis, see Figure 1.4) with other cell-free extracts that contains free molybdenum cofactor e.g. *E.coli*, or plant. The *nit-1* mutant lacks the molybdenum co-factor but, still retains the structural gene for NR enzyme and synthesises a protein

therefore, with NADPH cytochrome-C reductase but not NR activity. This mutant protein of *N. crassa* was found to sediment at 4.5s (roughly equivelent to that of *Aspergillus* group discussed above), whilst the wild-type *N. crassa* NR was found to sediment at 7.9s (similar to wild-type *A. nidulans*). When cell-free extract of the *N. crassa nit1* mutant was mixed with a source of Mo-Co such as *E.coli* cell-free extracts that contains free molybdenum cofactor), both the cofactor and the *nit-1* protein associated together to form the 7.9s species which then possesses both NADPH NR and cytochrome-C reductases activities. The addition of molybdate to the complementation mixture led to the stimulation of NR activity, such *in vitro* nitrate reductase was found to be identical to the native enzyme in terms of molecular weight, sedimentation coefficient and substrate affinities (Amy and Rajagopalan, 1979).

Nitrate reductase transfers two electrons from NADPH to nitrate through three redox centers composed of two prosthetic groups (Flavin adenine dinucleotide [FAD] and heme) and a Mo-Co. Each center is associated with a functional domain of the enzyme and has an activity independent on the other domains. When the alternative electron acceptor (i.e. cytochrome-C) is used instead of nitrate cytochrome-C reductase activity involves the transfer of electrons from NADPH to FAD, and reduces the heme domain, which in turn leads to the reduction of cytochrome-C. This infers that more than two domains (FAD and heme) are functional in this reaction, while the Mo-Co (the third domain) is not involved for the reaction

to function (Crawford *et. al.*, 1988; reviewed by Campbell and Kinghorn, 1990; Crawford, 1995).

Genetic and biochemical research studies on *A. nidulans* NR demonstrated that this enzyme is a dimeric composed of two components, the Mo-Co and a polypeptide encoded by the *niaD* gene. Two *niaD* gene products believed to form a complex with the Mo-Co, in order to give the dimeric enzyme (Cooley and Tomsett, 1985; reviewed by Cove, 1979) as discussed before.

Nitrate reductase has been purified from both fungal species, *Neurospora. crassa* and *Aspergillus. nidulans.* In *A. nidulans* Downey and Focht (1974) isolated a protein of 49 KDa. In later research work, Downey and Steiner (1979) purified polypeptides with subunit molecular weights of 49, 50, and 75 KDa. During yet another study, Steiner and Downey (1982) identified a polypeptide having a molecular weight of 54 KDa, suggesting that NR has a homotetrameric structure. Later, Minagawa and Yoshimoto (1982) have calculated the *A. nidulans* NR molecular weight, from sedimentation coefficients, to be 180 KDa and consisting of two subunits of 59 and 38 KDa. After the application of the rapid purification method developed for *N. crassa* (Horner, 1983). Cooley and Tomsett (1985) reported a subunit size of 91 KDa polypeptide, for *A. nidulans niaD* gene product of (i.e. the subunit polypeptide of NR). Since then using molecular cloning and DNA sequencing Johnstone *et. al.*, (1990) showed that the *niaD* gene encodes a protein

of 873 amino acids with a subunit size of 95 KDa. These molecular findings led to the suggestion that the *A. nidulans* native NR is a homodimeric structure with a 180 KDa molecular weight, and a subunit size of 95 KDa (the *niaD* gene product) these findings were found to be consistent with the original biochemical work of Minagawa and Yoshimoto (1982).

The *N. crassa* NR which is encoded by the *nit-3* gene, has been shown to have a molecular weight of 228 KDa (Garrett and Nasson, 1969) with a subunit sizes of 115 and 130 KDa. One of these two subunits was presumed to be a degradation product of the other (Pan and Nason, 1978). Later research work on *N. crassa*, and after the application of the rapid purification technique and cells extracted in the presence of high concentration of protease inhibitors, Horner (1983) finally reported a subunit size of 145 KDa for *N. crassa* NR enzyme.

The alga *Chlorella* NR protein structure has been widely studied, and found to be a homotetrameric enzyme. this is stable and easy to purify. The *Chlorella* NR was found to have a molecular weight of 360-380 KDa with four 90-100 KDa subunits. Each has been shown to contain a full set of prosthetic groups (Solomonson and MaCreery, 1986).

In the higher plants *Nicotiana tobacum* and *N. plumbaginifolia*, only the NADH form of NR (which is found to be dominant) has been isolated. In *Hordium vulgare* both forms NADH and NAD(P)H NRs have been isolated and the (NADP(H) form was found in all plant tissues, especially in roots. It has been reported that the nuclear-encoded NR of higher plants, that present in the cytoplasm in small amounts, was highly labile and difficult to purify. Plant NR has been reported to be a homodimer with a subunit size of 100-120 KDa, depending on the plant species. Each subunit contains one molecule of each of the prosthetic group (Pelsy *et. al.*, 1991). In contrast, both forms NADH and NAD(P)H NRs which present in rice and were detected in the leaves of the wild-type seedlings (Shen *et. al.*, 1976).

It has been reported that the *N. crassa* nitrite reductase (NiR) has a homodimeric structure of 290 KDa molecular weight, with two identical subunits each of 140 KDa. NiR enzyme has been shown to catalyse a 6 electron transfer from NADPH to nitrite *via* FAD, an iron-sulphur centre, and a siroheme cofactor (for a review see Dunn-Coleman *et. al.*, 1984 and references therein).

In *N. crassa* the NiR enzyme has been purified. The enzyme has been demonstrated to be a homodimer of 140 KDa subunits containing a siroheme prosthetic group which is thought to serve as a binding site and site of nitrite reduction (Prodouz and Garrett, 1981).

In bacteria, it has been shown that NiR uses NADPH as an electron donor, where the enzyme found to be a homodimer with a polypeptide of 88 KDa (Cole, 1989).

In higher plants, it has been demonstrated that the NADH nitrite reductase which is present in the chloroplasts, but encoded by a nuclear gene, uses reduced ferredoxin (Fd) as the electron donor. The (Fd) NiR was found to consist of a momomeric protein of 63 KDa (Back *et. al.*, 1991).

1.4.2 Genetics.

Mutations in the *A. nidulans* nitrate reductase structural gene (*niaD in A. nidulans*) resulted in strains resistant to chlorate and unable to utilise nitrate as the sole nitrogen source. In contrast, such mutants showed the wild-type phenotype on other nitrogen sources including nitrite the product of NR activity. Mutations in the *cnx* genes (ie. *cnxABC*, *cnxE*, *cnxF*, *cnxG*, and *cnxH*) leads to the loss of both nitrate reductase (NR) and purine hydroxylase (PH) activities (formerly xanthine dehydrogenase see section 1.2.2), due to the impairment in the biosynthesis of the molybdenum co-factor (Mo-Co) common to both NR and PH enzymes. Mutations in the *niiA* gene the structural gene for NiR leads to the loss of enzyme activity, which is required for the reduction of nitrite to ammonium *vis-a-vis* the nitrate assimilation pathway. Such mutants retain NR activity, and are unable to utilise both nitrate and nitrite as sole nitrogen source.

In contrast to *crnA*, mutants *niaD*, and *cnx* mutants *niiA* mutants are sensitive to chlorate (reviewed by Cove, 1979; Kinghorn and Unkles, 1994).

The structural gene for NiR has been identified in both *A nidulans* (*niiA*) and *N. crassa* (*nit-6*) (Tomsett and Garrett, 1980; reviewed by Cove, 1979; Dunn-Coleman *et. al.*, 1984). Nitrite reductase defective mutants in both *A. nidulans* and *N. crassa* are unable to utilise nitrate or nitrite as their sole nitrogen source. NiR mutants lack NiR enzyme, but have normal levels of nitrate reductase activity. When supplied with nitrate such mutants excrete nitrite (Tomsett and Garrett, 1980; reviewed by Cove, 1979).

1.4.3 Molecular Biology.

A large number of eukaryotic NR genes have been isolated and sequenced, including the NR structural gene (*niaD*) for *Aspergillus nidulans*. From the deduced protein sequences obtained from different eukaryotic NRs, amino acid similarities were observed between NRs from different eukaryotic organisms, and between NRs and other redox enzymes. Human and bovine NADPH cytochrome b_5 reductase, and spinach ferrodoxin NADP reductase showed similarity to the C-terminal FAD / NADPH or cytochrome b reductase domain. The central heme domain showed 13 complete identities with all members of the cytochrome b_5 superfamily. The N-terminal Mo-Co NR domain has shown homology with the rat sulphite oxidase, and

with xanthine dehydrogenase from the fruit fly *Drosophila* (Rouze and Caboche, 1992; reviewed in Kinghorn and Campbell, 1989).

Molecular studies of nitrite reductase cDNA clones from both spinach and corn have revealed that the polypeptide of the spinach enzyme has a bout 600 amino acid residues, while the *E. coli* and *Aspergillus nidulans* NiRs has 838 and 1104 residues, respectively (Back *et. al.*, 1991; reviewed by Kinghorn and Campbell, 1989).

1.5 Molybdenum Cofactor Biosynthesis.

1.5.1 Biochemistry.

Mo-Co deficient mutants in both prokaryotic (bacteria) and eukaryotic (algae, fungi, mosses, insects, humans, and higher plants) organisms were found to be unable to utilise nitrate or hypoxanthine as the sole nitrogen source (reviewed by Wray and Kinghorn, 1989) and see section 1.5.2. Some of the genes that have been suggested to be involved in Mo-Co biosynthesis have been sequenced in both prokaryotes and eukaryotes. On the bases of the obtained high homologies in the sequences between the equivalent genes from different organisms whether prokaryotes or eukaryotes, in addition to the biochemical results Mo-Co genes have been classified into 4 major groups. The first group, is involved in the early steps of the Mo-Co biosynthesis (Rivers *et. al.*, 1993; Heck and Ninnemann, 1995; Stallmeyer *et. al.*, 1995). The second group, plays a role in sulphur

donation to the so called converting factor (Pitterle *et. al.*, 1993; Wuebbens and Rajagopalan., 1993; Heck and Ninnemann, 1995; Wuebbens and Rajagopalan., 1995). The third group, is required for molybdenum transport (the uptake and release) in the cell (Rech *et. al.*, 1995). The final group. has been shown to be involved in the insertion of molybdenum into the molybdopterin molecule (Hinton and Dean, 1990; Heck and Ninnemann, 1995) see section 1.6.

The biochemical properties of the temperature-sensitive mutations provide clear evidence for the roles of the *A. nidulans cnx* genes in the nitrate reductase and purine hydroxylases I and II activities. Temperature sensitive mutations are probably due to a very small change in a given protein, which shows function at the permissive temperature (i.e. non-selection temperature) but not at the non permissive. If the product of a given *cnx* gene is a small protein or peptide, structurally involved in the biosynthesis of the cofactor, then certain mutations in such a gene may result in a temperature-sensitive growth phenotype which, should result in the enzyme being active when the mutant is grown at the permissive temperature, at which the enzyme should be more heat labile than the wild-type. Furthermore, if the *cnx* gene is specifying a non-structural part of an enzyme, the thermal stability in the mutant should be the same as in the wild-type at the permissive temperature (MacDonald and Cove, 1974; reviewed by Cove, 1979).

A cryso-sensitive or cold-sensitive *cnxC* mutant (designated *cnxC20*) was isolated by Arst and colleagues (1982). This was found to grow normally at 37°C and showing similar growth properties to *cnxJ1* and *cnxJ2* strains. The similar growth properties shown at 37°C by these different mutants are including a wild-type growth on nitrate, hypoxanthine, nicotinate, and scarcely utilising nicotinate or hypoxanthine in the presence of allopurinol, while showing sensitivity to nitrate in the presence of 33 mM molybdate. At 25°C the *cnxC20* mutant had a phenotype similar to a totally mutant *cnx* (i.e. no growth on nitrate and hypoxanthine), which is different from the *cnxJ1* mutants. The *cnxC20* mutant possessed extremely heat labile NR which lead to the suggestion that the product of this gene i.e. *cnxC* might be also a structural component of the NR enzyme. Since the cofactor is more likely to have a low molecular weight, so it is therefore difficult to predict that the NR enzyme could have another polypeptide component in addition the component which might be specified by *cnxH* (MacDonald and Cove, 1974; Scazzocchio, 1980; Arst *et. al.*, 1982; reviewed by Cove, 1979).

As for *cnxC*, the properties of *cnxH* temperature-sensitive mutants (namely *cnxH11*, *cnxH17*, and *cnxH21*) (MacDonald and Cove, 1974) indicated that NR enzyme is a temperature-liable and markedly unstable one compared to that of the wild-type. This lead to the suggestion that the *cnxH* gene might specify a protein or polypeptide structurally required for nitrate reductase activity. Additionally, previous results presented by MacDonald and Cove

(1974) showed that temperature-sensitive *cnxH* mutants (*cnxH11*, *cnxH17*, and *cnxH21*) were totally phenotypically mutant (i.e. did not show any kind of growth on hypoxanthine at either the permissive or the non-permissive temperature) with respect to hypoxanthine, i.e., having NR but not PHI and II activities at 25°C. The *cnxE* (*cnxE35*, *cnxE36*), and *cnxF* (*cnxF21*, *cnxF24*) temperature-sensitive mutants had the activity of the three molybdoenzymes at low temperature (25°C). These *cnxH* findings can only be explained if the product of *cnxH* gene is considered as a structural component for all three molybdoenzymes in the assimilation pathway, although there is no strong direct evidence for this, so far. Therefore, there is a strong suggestion that *cnxH* gene codes for a NR associated polypeptide component, into which the cofactor (that has been specified by other *cnx* genes) is attached (MacDonald and Cove, 1972; MacDonald and Cove, 1974; MacDonald *et. al.*, 1974; Scazzocchio, 1974; Cove, 1979; Arst *et. al.*, 1982).

In contrast, enzyme extracts from mutants strains *cnxE35*, *cnxE36*, *cnxF21*, and *cnxF24* (MacDonald and Cove, 1974) phenotypic temperature-sensitive mutants showed heat liability of NR similar to that of the wild-type, which indicates that the products of these genes might specify an enzyme responsible for the insertion of molybdenum into the cofactor, specified by other *cnx* genes i.e. not NR structurally involved. This suggests that both *cnxE* and *cnxF* genes have catalytic functions in the biosynthesis of the cofactor (Arst

et. al., 1970; Cove, 1970; MacDonald and Cove, 1972; MacDonald and Cove, 1974; MacDonald *et. al.*, 1974; Scazzocchio, 1974; Arst *et. al.*, 1982).

1.5.2 Genetics.

Amongst mutants of *A.nidulans* which have been isolated for their inability to utilise nitrate as sole nitrogen source, mutants map in at least seven unlinked loci in *A. nidulans*, in which they lack detectable NADPH-nitrate reductase activity. These loci include the *niaD* locus which maps in linkage group VIII (*niaD* mutants can utilise hypoxanthine, but not nitrate), which is the structural gene for the enzyme nitrate reductase (NR). Additionally, the *nirA* locus maps in linkage group VIII, its product necessary for the regulation of enzyme expression in nitrate assimilation pathway (see section 1.2.2). In addition, five unlinked loci designated *cnx* (**c**ofactor for **n**itrate reductase and **x**anthine dehydrogenase) distributed through over chromosomes II, III, VI, VII, and VIII (for the location of these *cnx* on the *A. nidulans* linkage map see below). Mutation within the last five (*cnx*) loci results in the inability of the mutant to utilise either nitrate or hypoxanthine (substrates for the three molyboenzymes) as sole nitrogen source. Loss of function in any of these loci products leads to loss of activity of the three molybdoenzymes, namely nitrate reductase, purine hydroxylase I and II (formerly named xanthine dehydrogenase I and II). Such loss of activity was suggested to be due to the lack of Mo-Co biosynthesis, the enzyme steps being specified by the five *cnx* loci and required for the activity of these enzymes.

Heterokaryon complementation tests for nitrate utilisation between the *cnx* mutants revealed that, these mutants fall into originally thought to be seven complementation groups, designated: *cnxA*, *cnxB*, *cnxC*, *cnxE*, *cnxF*, *cnxG*, and *cnxH*, (their function and genotypes are illustrated in Table 1.2). In fact these are only five unlinked loci, since *cnxA*,B and C form one complex locus termed *cnxABC* (Cove, 1963; Pateman *et. al.*, 1964; Darlington *et. al.*, 1965; Pateman and Cove, 1967; Cove and Pateman, 1969; Arst *et. al.*, 1970; MacDonald and Cove, 1974; MacDonald *et. al.*, 1974; Scazzocchio, 1974; Garrett and Cove, 1976; Cove, 1979; Scazzocchio, 1980; Arst *et. al.*, 1982).

Mutations with the *cnxABC* locus which maps in linkage group VIII (Figure 1.3), showed complex overlapping pattern of complementation. First, *cnxA* and *cnxC* mutants complement each other. Second, a class of mutants failed to complement both *cnxA*, and *cnxC* mutants. These were designated *cnxB* mutants. Mutants which failed to complement *cnxA* and B but not C were classified as *cnxA* mutants, while mutants which complement both *cnxA* and *cnxB* but not *cnxC* were classified as *cnxC* mutants. Since all three classes are genetically closely linked it was not clear if this *cnxABC* locus contains a single gene where mutations fall into three intracistronic complementation groups, two contiguous genes viz*cnxA* and *cnxC* with *cnxB*$^{-}$ being as a result of a double or a deletion mutation (Arst and Cove, 1970; Scazzocchio, 1980; Arst *et. al.*, 1982; reviewed by Cove, 1979).

Table 1.2 Genes Involved In Nitrate Assimilation.

Gene	Proposed Function	Phenotype Of Mutants	Reference(s)
areA	Positive-acting regulatory gene whose product is required for nitrogen metabolite repression of nitrate assimilation system. product inactivated by ammonium. Recently, the gene was found to encode protein with a putative zinc finger which shown to have a regulatory role.	Chlorate resistant, grows on ammonium, its product necessary for the synthesis of ammonium repressible activities. *areA* ^{r} : able to utilise ammonium, but not many other nitrogen sources. *areA* ^{d} : the growth as the wild-type, derepressed for some normally ammonium-repressible activities.	Arst and Cove (1973). Kudla *et. al.*, (1990)
cnxABC	Product is structural component of an enzyme involved in the synthesis of the molybdenum cofactor required for nitrate reductase, purine hydroxylase I and II activity (Formerly named xanthine dehydrogenases I and II.	Can not use either nitrate or hypoxanthine as sole nitrogen source, able to use nitrite and uric acid .	Pateman *et al.*, (1964) Pateman *et al.*, (1967) MacDonald and Cove (1974) Scazzocchio (1974).

Table 1.2 Continued.

Gene	Proposed Function	Phenotype Of Mutants	Reference(s)
cnxE	Specifies an enzyme responsible for the incorporation of molybdenum into the enzyme, but not structurally involved. (Catalytic function in biosynthesis of cofactor).	As *cnx ABC* and partially repairable for growth on nitrate by the addition of 33 mM molybdate to the medium.	As for *cnxABC*
cnxF	As for *cnx E*	As for *cnx ABC*	As for *cnxABC*
cnxG	As *cnx ABC*	As *cnx ABC*	As for *cnxABC*
cnxH	Specifies a protein or polypeptide structurally involved in nitrate reductase.	As for *cnx ABC*.	As for *cnxABC*
cnxJ	Might has a role in the regulation of the cofactor biosynthesis.	Can not use hypoxanthine as nitrogen source .	Arst and Cove (1969, 1973) Arst *et al* (1982).
crnA	involved in nitrate transport.	Chlorate resistant grows on nitrate and hypoxanthine as nitrogen sources.	Cove (1979). Tomsett and Cove (1979).

Table 1.2 Continued.

Gene	Function	Phenotype Of Mutant Alleles	Reference(s)
meaB	The *meaB* gene is involved in methylammonium and ammonium transport, and in their efflux. Also it might be involved in nitrite uptake. Recently, the gene found to encode for a novel protein product which might be a new transcriptional regulatory protein.	*mea* ^{R} able to use nitrate in the presence of high concentrations of methylammonium, the toxic analogue of ammonium (gives methylammonium-resistant phenotype). Also have NR and NiR activity in the presence of ammonium, although still requiring nitrate for induction. *meaB*: Intracellular levels of methyammonium are lower than wild-type, If ammonium levels were similarily depressed lead to mutants ammonium-derepressed phenotype. The basis of their ammonium-derepressed phenotype are unknown. It is hypersensitive to nitrite.	Arst and Cove (1969) (reviewed by Cove, 1979) Polley and Caddix (1996)
niaD	Structural gene for nitrate reductase.	can not use nitrate but can use nitrite as sole nitrogen source	Pateman *et al* (1964) MacDonald and Cove (1974).

Table 1.2 Continued.

Gene	Proposed Function	Phenotype of mutants	Reference(s)
niiA	Structural gene for nitrite reductase enzyme.	Can not use both nitrate and nitrite but with ammonium it can.	Pateman *et al* (1967).
niiC628	Mutation leads to nitrite hypersensitivity, which leads to altered permease function by either increasing nitrite uptake or decreasing the efflux.	Grows with a nitrogen-starved morphology on nitrate or nitrite but, has the wild-type levels of NR and NiR. Grows normally on all nitrogen sources but, unable to grow on mixture of urea and nitrite.	(reviewed by Cove, 1979)
nirA	Regulatory gene for the induction of both nitrate and nitrite reductase reduction. Recently, the gene was found to encode protein with a putative zn(II)2-Cys6 cluster DNA binding site.	Its product required for the induction of *niaD and niiA* genes, only active in the presence of nitrate. *nirA*$^{-}$: unable to utilize nitrate or nitrite as nitrogen source. *nirA*^{c} : mutants synthesis nitrate and nitrite reductases constitutively.	Pateman and Cove (1967). Cove (1970). Burger *et. al.*, (1991 a, b)

Table 1.2 Continued.

Gene	Function	Phenotype of mutant alleles	Reference(s)
tamA	The product plays a role in mediating ammonium repression. May function similarily to *areA* gene, both gene products being necessary for the expression of the *niiA* and *niaD* genes. In addition, it might be involved in nitrite uptake. The exact function is unknown Recently, the gene was found to encode a protein with a putative zn(II)2-Cys6 cluster DNA binding domain (not required for function in *A. nidulans*)	*tamA* ^{r} : resistant to thiourea, aspartate, hydroxymate, and chlorate. In contrast, it is sensitive to nitrite.	Kinghorn and Pateman (1975). Davis *et. al.*, (1996)

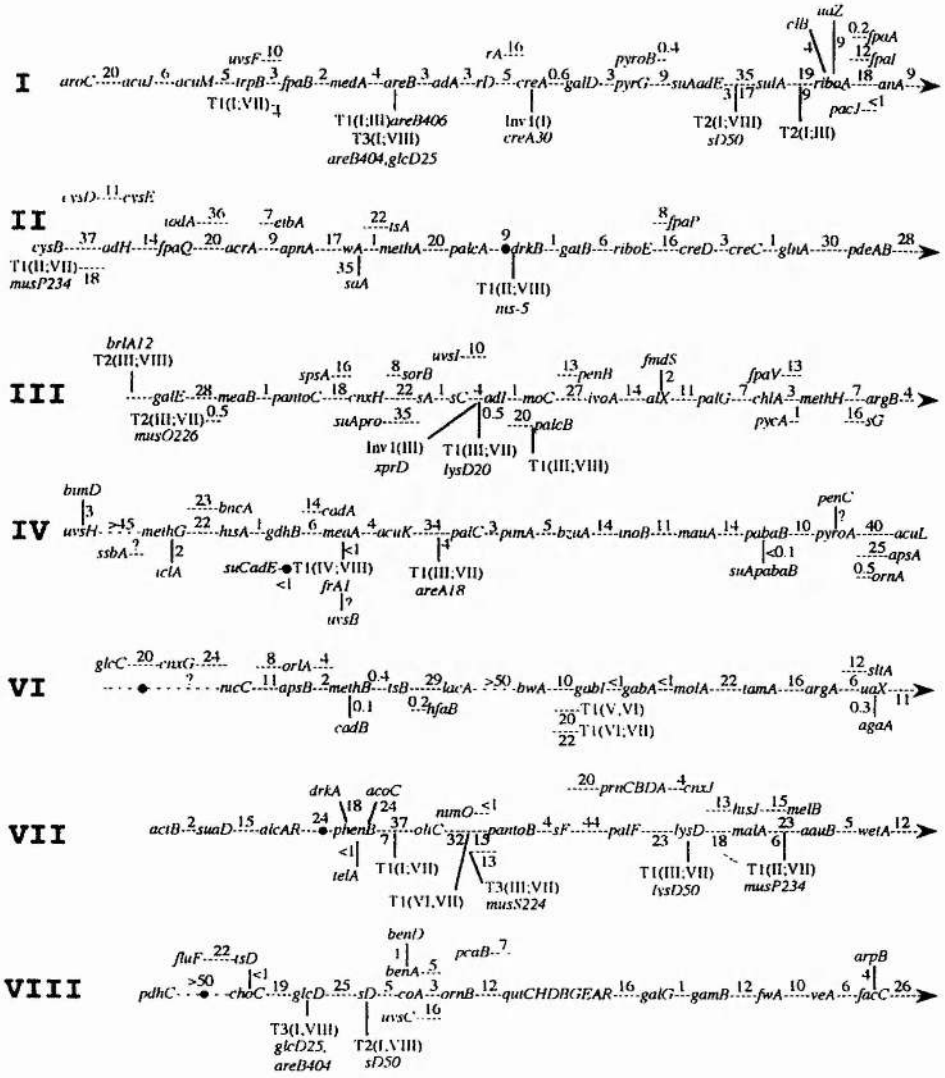
The *cnxE* mutants maps in linkage group II (Figure 1.3), it is thought that its product is concerned with the insertion of molybdenum into the cofactor (Arst *et. al.*, 1970; Cove, 1979; Arst *et. al.*, 1982).

The *cnxF* gene locates in linkage group VII, *cnxG* locates on linkage group VI, and *cnxH* is found on linkage group III (Figure 1.3), their function and phenotype are illustrates in Table 1.2 (MacDonald and Cove, 1974; MacDonald *et. al.*, 1974; reviewed by Cove, 1979).

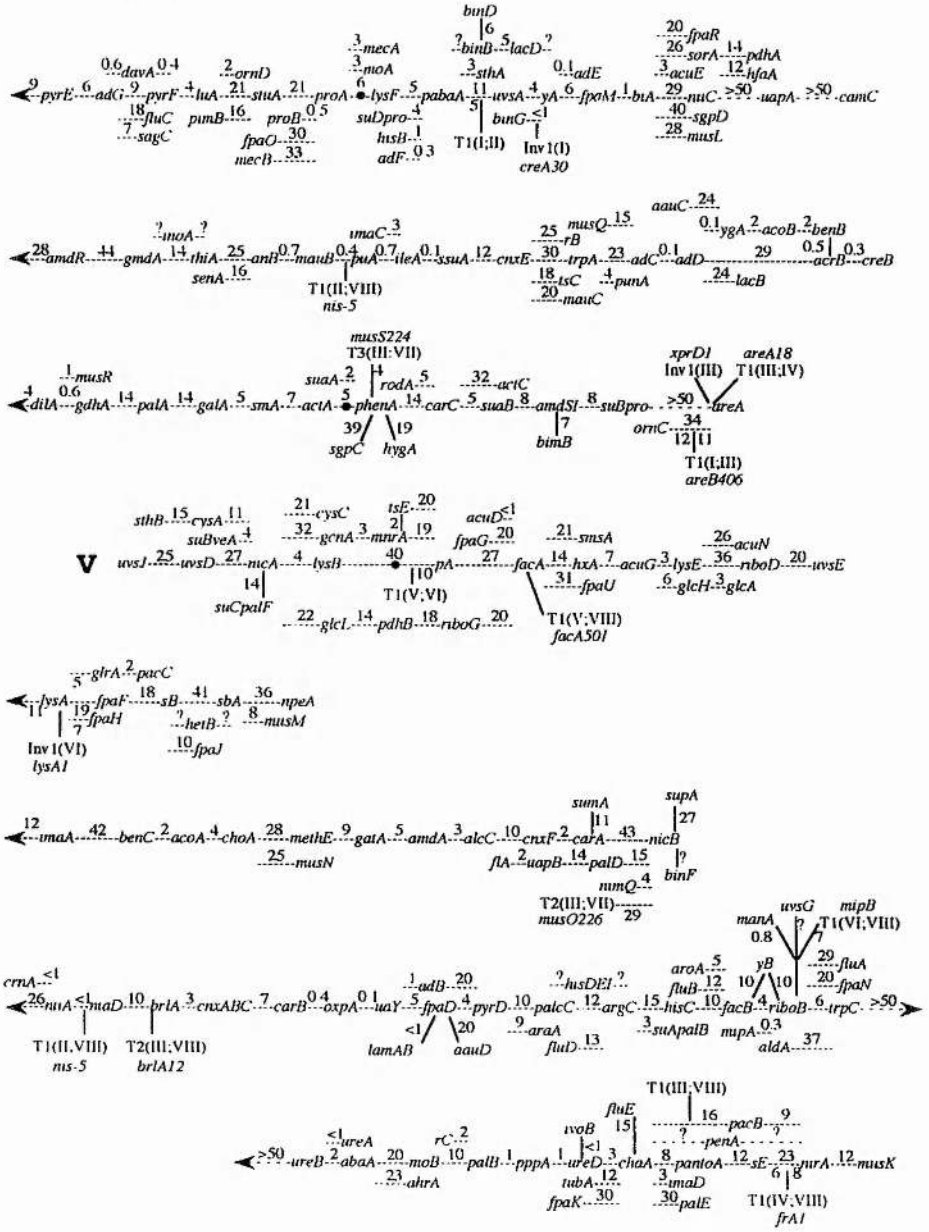
A further *cnx* locus in *A. nidulans* has been identified (Arst *et. al.*, 1982) designated *cnxJ* , maps in linkage group VII (Figure 1.3). Mutants in the *cnxJ* locus are different in terms of phenotype from the other *cnx* mutants. *cnxJ* mutants do not affect (at either 25°C or 37°C) the utilisation of nitrate or hypoxanthine as sole nitrogen source, unless tungstate (molybdate analogue) or methylammonium is present in the medium i.e. they fail to grow on nitrate or hypoxanthine in the presence of tungstate or methylammonium. No evidence is available which indicates that *cnxJ* mutations lead to a complete loss of any or all of the three molybdoenzymes judged by growth tests and enzymology. Instead the mutations, only seem to affect the levels of these enzymes. This suggests that mutations in the *cnxJ* locus effectively reduce the level of molybdenum-containing cofactor, when tungstate competes with molybdate at the uptake level, once inside the cell for incorporation into the cofactor.

Figure 1.3. The Linkage Map Of *Aspergillus nidulans*. The map indicating where the genes lie within linkage group. Map units are in centiMorgans (From Clutterbuck, 1994).

Linkage Map of



Aspergillus nidulans



This suggested to Arst and colleagues that the *cnxJ* gene might be involved in the regulation of cofactor production and thus was defined as a sixth *cnx* locus (Arst *et. al.*, 1982; reviewed by Cove, 1979).

1.5.3 Molecular Biology.

Molybdenum Cofactor.

Molybdenum is regarded as an essential trace element for most if not all the organisms. In order for it to be a catalytically active metal in molybdoenzymes activity, it has to be complexed to a reduced pterin species termed molybdopterin, thus forming the molybdenum co-factor (Mo-Co). This cofactor is an essential and a common component required for the activity of several molybdoenzymes, including NR, PH, sulphite oxidase, aldehyde oxidase, and formate dehydrogenase in both prokaryotic and eukaryotic metabolic pathways such as ones required for nitrate assimilation, sulphur or purine metabolism (Amy and Rajagopalan, 1979; Johnson, 1980; Rajagopalan and Johnson, 1992) or human hormones biosynthesis (Wadman *et. al.*, 1983; Johnson *et. al.*, 1989; Bamforth *et. al.*,1990). Additionally, Mo-Co appeared to have a similar structure in all organisms investigated thus far. This makes it likely universal in function, forming part of a catalytically active centre for different enzymes whose products are necessary for the viability of all organisms, whether prokaryotes or eukaryotes (Amy and Rajagopalan,1979; Johnson *et. al.*, 1984; Kramer *et. al.*, 1987; Johnson *et. al.*, 1989; Bamforth *et. al.*,1990; Rajagopalan and Johnson, 1992; Hoff *et. al.*, 1995; reviewed in Johnson, 1980).

Molybdenum-cofactor deficient mutants involved in the regulation of the cofactor biosynthsis were described previously in bacteria, fungi, algae, mosses, higher plants, insects (Wray and Kinghorn, 1989 and references therein), and humans (Wadman *et. al.*, 1983; Bamforth *et. al.*,1990). In *A. nidulans* there are five loci (designated *cnx*) involved as discussed above (Pateman *et. al.*, 1964; MacDonald *et. al.*, 1974; Arst *et. al.*, 1982). In the related fungus *N. crassa* five similar loci (designated *nit*) exist (Amy and Rajagopalan,1979; Heck and Ninnemann, 1995). In higher plants, six loci (again designated *cnx*) (Stallmeyer *et. al.*, 1995; Hoff *et. al.*, 1995) are present. In bacteria five loci (designated mo, formerly named *chl* genes) (Amy and Rajagopalan,1979; Johnson, 1980; Johnson *et. al.*, 1984; Johnson *et. al.*, 1989; Rajagopalan and Johnson, 1992). The molybdenum cofactor is essential for the activity of all molybdoenzymes. Since molybdenum cofactor genes play a role in its biosynthesis, mutations at any of the biosynthetic loci leads to a loss of molybdoenzyme activity (Pateman *et. al.*, 1964; Darlington *et. al.*, 1965; Scazzocchio, 1973; MacDonald *et. al.*, 1974; Arst *et. al.*, 1982).

In humans, deficiency in molybdenum cofactor levels came as a result of a defect in molybdenum incorporation or utilisation. Combined deficiences of several molybdoenzymes, such as sulphite oxidase, xanthine dehydrogenase, and aldehyde oxidase led to neurological abnormalities, mental retardation, dislocation of optical lenses and even death at a young age (Wadman *et. al.*, 1983; Bamforth *et. al.*,1990). Several cases in molybdenum cofactor deficiency have

been reported for humans, where it has been possible to identify two genetic complementation groups (i.e. A and B) in humans culture cells obtained from see below affected patients. Complementation group A, found to be analogous to *moaA* (Johnson *et.. al.*, 1989) exhibit converting factor activity when supplied with a source of the precursor i.e. the gene product participates in converting guanosine into precursor Z . Complementation group B, found to be analogous to new locus designated *moeB* of *E. coli* mutant which is responsible for creating a reactive sulphur on the small subunit of the converting factor, by that accumulate the molybdopterin precursor (Wadman *et. al.*, 1983; Johnson *et. al.*, 1989, Bamforth *et. al.*,1990).

1.6 Related Genes For Molybdenum Cofactor In Other Organisms.

Not unexpectedly, the Mo-Co system in *E coli* has been extensively studied (reviewed in Rajagopalan and Johnson, 1992). Chlorate resistant *E. coli* mutants and defective in Mo-Co biosynthesis have been isolated, mapped genetically and found to lie in five different loci. These are now known as *moa*, *mob*, *mod*, *moe*, and *mog* (formerly named *chlA*, B, D, E, and G respectively). From the results of biochemical studies using different Mo-Co *E.coli*, mutants a model of the cofactor biosynthetic pathway was proposed (Figure 1.4). Each locus has been found to encode one or more enzymes involve in the Mo-Co biosynthetic pathway. The number of genes in each locus varies from one to five. Five tightly linked genes designated *moaA*, B, C, D, and E were found to comprise an operon in

the *moa* locus. Four genes (*modA*, B, C and D) were found to be parts of the *mod* operon. Two genes make up *mob* locus, and a single gene only is found in the *mog* locus (*mogA*) (Hinton and Dean, 1990; Hoff *et. al.*, 1995; Stallmeyer *et. al.*, 1995).

Two genes *moa* and *moe* were found to be required for the early steps (i.e. molybdopterin biosynthesis) (Figure 1.4). The DNA sequence for both genes has been determined (Rivers *et. al.*, 1993) and the gene products of *moaA*, B, and C were thought to be involved in the production of the molybdopterin precursor (Johnson and Rajagopalan, 1987).

The genes *moaD* and *moaE* encode two proteins which form a heteromeric converting factor which has a role in converting the molybdopterin precursor (i.e. precursor Z) into molybdopterin (Rivers *et. al.*, 1993; Pitterle and Rajagopalan, 1993).

In contrast to eukaryotes the *E coli* Mo-Co has been reported to contain a GMP moiety, attached to the molybdopterin to form molybdopterin guanine dinucleotide (termed MGD). The molecule was found to be present in *mob* mutants (Johnson *et al.*, 1991). The *mob* locus (Figure 1.4) has a catalytic function which catalysis the synthesis of an active MGD from MPT (Johnson *et. al.*, 1991; Hoff *et. al.*, 1995; Stallmeyer *et. al.*, 1995). The *mob* mutants were found to be lack a protein called FA. It has been reported that FA production occurred in all Mo-Co mutants with the exception of *mob* strains.

This observation led to the suggestion that the FA protein itself is the *mob* gene product (Low *et. al.*, 1988). Recently, Labbi-Nivol *et. al.*, (1995) reported that the purified FA protein can activate *in vitro* inactive molybdoenzymes in *mob* mutant cell extracts. Very recently, Palmer *et..al.*, (1996) reported that *mobB* gene is not essential for Mo-Co biosynthesis because a deletion of both *mob* genes can be fully complemented by just *mobA*, i.e. inactive NR purified from both *mob* strains can be activated *in vitro* by incubation with *mobA* gene product (i.e. protein FA), GTP, $MgCl_2$ and a protein fraction designated factor X. Results obtained indicate that factor X activity was present in strains that lack *mobB* i.e. *mobB* gene is not essential for the activity of this factor but, over expression of *mobB* product would lead to increased levels of the factor X. These data would suggest that the *mobB* gene can participate in NR activation, whereas, factor X was found to be the gene product of *narJ* gene. This supported the idea that *E. coli. mobA* gene encodes the FA protein. Whilst *mobB* gene encodes a polypeptide with a putative binding site, which is suggested to be the site for binding guanine nucleotide required for the synthesis of molybdopterin guanine dinucleotide (Santini *et. al.*, 1992). The *E. coli mob* gene has not been isolated thus far. the *mod E. coli* mutants are thought to be analogous to the *Aspergillus. nidulans cnxE* mutants since they showed phenotypic repairability on nitrate in the presence of molybdate (50 mM) in the medium (Glaser and Demoss, 1971).

The *E. coli* molybdopterin-deficient *moeB* mutants have been shown to accumulate large amounts of precursor Z, the final intermediate in molybdopterin biosynthesis. The accumulation of the precursor in such mutants led to the suggestion that, *moe* (Figure 1.4) locus has a role in sulphur donation to the converting factor, which is an enzyme known to open the cyclic phosphate of precursor Z, and introduces a diththiolene into the side chain to build up MPT (Pitterle *et. al.*, 1993; Wuebbens and Rajagopalan, 1993; Wuebbens and Rajagopalan, 1995).

Recently, the *mod* operon (consisting of *modA*, B, C, D genes) has been sequenced (Maupin-Furlow *et. al.*, 1995) and shown to encode molybdenum specific transport systems (Figure 1.4), which mediate the uptake and the release of molybdenum into cells. The regulation of the *mod* operon in *E. coli* was found to be controlled by availability of molybdenum in the medium (Rech *et. al.*, 1995). In addition to the *mod* specific system for molybdenum transport sulphate system can be used under conditions of unavailability of the *mod* system. A third non-specific molybdenum transport system has been shown to be present in *E. coli* where, it became active under conditions of high molybdate concentrations (Rosentel *et. al.*, 1995).

Finally, the *E. coli* *mogA* gene (Figure 1.4) product (formerly *chlG*) has been shown to be involved in the insertion of molybdenum into molybdopterin compound (Hinton and Dean, 1990).

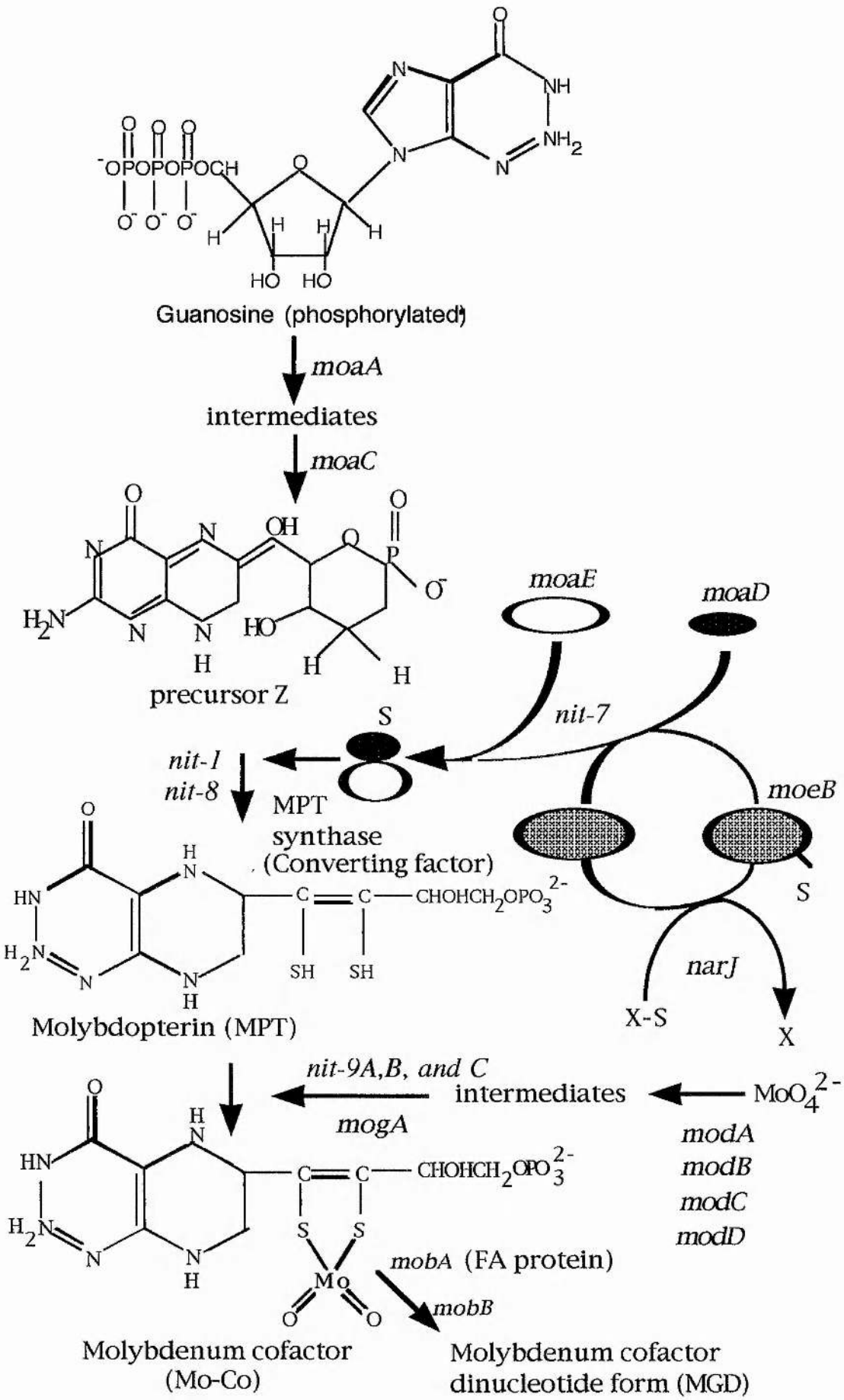
Very recently biochemical analysis of MPT levels (Joshi *et. al.*, 1996) in wild-type *E. coli* and in the *mod* and *mog* mutants indicate that *E.coli* cells in absence of added molybdate were containing approximately 11% of the wild-type levels of MPT, whereas, in molybdate supplemented medium (1 mM) *mod* mutants were showing the wild-type levels. In contrast, *mog* cells were yielding approximately 15% of wild-type levels whether or not molybdate was added. These data may suggest hat both *mod* and *mog* MPT may be synthesised normally but subject to degradation if further assembled into Mo-Co and incorporation into molybdoenzymes is interrupted. However, the failure of *mod* mutants to incorporate molybdenum and assemble Mo-Co can be explained by failure in function of one or more of the *mod* operon genes. Furthermore, the failure of *mog* mutants to incorporate and assemble Mo-Co is not reversed by molybdate supplementation which would suggeste that *mog* gene may function as a molybdochelatase (an enzyme required for the assembly of Mo-Co from MPT in presence of low molybdate concentrations.

Biochemical characterisation of *Neurospora crassa* molybdenum wild-type and mutants (i.e. *nit-1*, *nit-7*, *nit-8*, and *nit-9A*, *nit-9B* and *nit-9C*) demonstrated the presence of converting factor activity as well as its substrate, precursor Z (Figure 1.4) (Heck and Ninnemann, 1995). The *nit-1* mutant was found to be able to synthesise precursor Z, the direct precursor of MPT but, it was found not able to convert this precursor into MPT. As for *nit-1*, mutant *nit 8* has been defined as a further gene required for converting factor

activity, by its inability to use precursor Z of *nit-1* mutant (Figure 1.4). This inability lead to the suggestion that precursor Z is made up of different subunits encoded by the two different genes separately. Biochemical experiments indicated that the mutant *nit-7* of *N. crassa* is free of precursor Z but was able to build MPT from precursor Z by itself. This ability of *nit-7* mutant suggests that the mutant possesses the converting factor (Figure 1.4). Such enzymes were found to be able to provide the converting factor with sulphur. The *nit-9* A, B, and C mutants of *N. crassa* were found to have a protein-bound precursor form of Mo-Co, which suggests that MPT is bound to apo-NR. This MPT is converted into active Mo-Co (Figure 1.4) in the presence of high molybdate concentrations (Heck and Ninnemann, 1995) in this mutant.

Following NTG mutagenesis very recently Golden and co-workers have isolated six mutants of *Anabaena* an alga that forms heterocysts when grown on nitrate. One of these mutants (which found to form approximately 6% to 8% heterocysts) was complemented by a 1.8kb chromosomal DNA. Sequence analysis indicate that 1.2kb of the whole fragment was an ORF designated *moeA* and the deduced amino acid sequence of this *moeA* polypeptide showed approximately 37% identity to the *E. coli moeA* gene which is suggested to be required for the synthesis of molybdopterin cofactor (Ramaswamy *et. al.*, 1996)

Figure 1.4. Proposed Model For Mo-Co Biosynthesis In *E coli* (A Modified Form Of That Presented By Stallmeyer *et. al.*, 1995,). Guanosine is converted into precursor Z by *moaA* and *moaC* gene products. This the precursor is transformed into molybdopterin {MPT} by the (MPT synthase) converting factor which is encoded by *moaD* and *moaE*, and activated by a sulfotransferase encoded by *moeB*. Factor X is the gene product of *narJ* gene (Palmer *et. al.*, 1996). Molybdenum uptake and release into the cell is mediated by the *mod* operon. The *mogA* gene product is responsible for the insertion of molybdenum into molybdopterin {MPT}. The *mob* locus catalysis the synthesis of active MGD from MPT, *mobA* gene encodes the FA protein which activates inactive molybdoenzyme (Palmer *et. al.*, 1996). *mobB* encodes a polypeptide with binding site suggested to be for binding guanine nucleotide required for MGD synthesis (Santini *et. al.*, 1992) i.e. The *mob* gene products are responsible for the addition of dinucleotide into the Mo-Co (present in bacteria but not in eukaryotes). No functions have yet been assigned to the *moaB* and *moeA* genes. In *N. crassa* the probably equivalent genes designated *nit* (for details about their roles see section 1.6).


Guanosine (phosphorylated)
moaA
intermediates
moaC
precursor Z
moaE
moaD
nit-7
S
nit-1
nit-8
MPT
synthase
(Converting factor)
moeB
S
narJ
X-S
X
Molybdopterin (MPT)
nit-9A,B, and C
mogA
intermediates
MoO4 2-
modA
modB
modC
modD
mobA (FA protein)
mobB
Molybdenum cofactor
(Mo-Co)
Molybdenum cofactor
dinucleotide form (MGD)

Molybdenum cofactor mutants have been studied extensively in the fruit fly *Drosophila melanogaster* in which mutants are easily identified by eye colour variations (wild-type red, and mutant white eye colour). Such colour variation is a result of deficient xanthine dehydrogenase activity. The *Drosophila* molybdenum cofactor gene so-called cinnamon has been isolated, sequenced and found to be homologous to three *E. coli* (*moeA*, *moaB* and *mogA*) molybdenum cofactor proteins, *Arabidopsis cnx1* protein and to the rat protein Gephyrin (Kamdar *et. al.*, 1994).

In higher plants Mo-Co deficient mutants have been isolated and located in 6 different complementation groups, (designated *cnxA*, *cnxB*, *cnxC*, *cnxD*, *cnxE* and *cnxF*). Such Mo-Co deficient plant mutants are inable to utilise nitrate as a sole nitrogen source due to a lack of NR enzyme activity (Hoff *et. al.*, 1995) similar to the phenotype of *A. nidulans* mutants. Valuable information about plants Mo-Co biosynthesis has been obtained by cloning the genes involved on the basis of complementation of *E. coli* mutants with plant cDNAs, basically by selecting for the restoration of ability of a given *E. coli* mutant to use nitrate as the terminal electron acceptor under anaerobic conditions (restored NR activity) (Hoff *et. al.*, 1995).

It has been demonstrated that in the higher plants *Arabidopsis thaliana,* and *Nicotiana plumbaginifolia* , mutants in the Mo-Co loci *chl6* and *cnxA*, respectively have shown to be partially molybdate repairable when grown on nitrate with high concentrations of

molybdate. It was suggested that these genes are involved in the insertion of molybdenum into molybdopterin most likely the last step in Mo-Co biosynthesis. This role resembles the function predicted for *A. nidulans cnxE* (Stallmeyer *et. al.*, 1995).

Two cDNA clones from the higher plant *Arabidopsis thaliana* have been isolated by functional complementation of two *E. coli* mutants (i.e. mutations *moaA* and *moaC genes*) which are deficient in single steps early in Mo-Co biosynthesis. Such successful gene cloning indicates a high degree of functional similarity in the Mo-Co biosynthesis in both prokaryotes and eukaryotes. The plant genes, and these genes were termed as *cnx2* and *cnx3*, and they showed not unexpectedly significant identity to *E. coli* *moaA* and *moaC* genes (Hoff *et. al.*, 1995).

A further *Arabidopsis thaliana* cDNA clone (designated *cnx1*) was also isolated by complementation of a *mogA* mutant and sequenced (Stallmeyer *et. al.*, 1995). The inferred protein sequence reveals that the product of this protein has a two-domain structure. This multifunctional protein exhibits homology to no less than three different Mo-Co biosynthetic *E. coli* proteins (ie. *moeA*, *moaB*, and *mogA*). The N terminal domain of the *cnx1* protein was found to be homologous to the *E. coli moeA* protein, whilst the C-terminal was homologous to *moaB* and *mogA* proteins. Additionally, this *cnx1* protein has shown to have similarity with the fruit fly (*Drosophila*) protein cinnamon, as well as to the mammalian (rat) neuroprotein,

Gephyrin). These eukaryotic homologous proteins also showed a two-domain structure, but the order of the domains is inversed as compared to the *Arabidopsis cnx1* domain (Stallmeyer *et. al.*, 1995).

1.7 Other Genes Which May Be Required For Nitrate Assimilation.

1.7.1 Chlorate Resistance.

A very large number of chlorate resistant mutants have been isolated from bacteria, fungi, algae, and higher plants (Wray and Kinghorn, 1989 and reviews therein). Most of these mutants were found to be defective in nitrate reduction due to mutations in structural genes for NR, Mo-Co biosynthesis regulation, or nitrate transport. Certain mutants were first identified for their inability to utilise nitrate (*crn* mutants showed wild-type growth on nitrate) as sole nitrogen source, while returing the ability to utilise nitrite and ammonium (ie *niaD* and *cnx* mutants) (Cove and Pateman, 1963; Pateman *et. al.*, 1964; Herman and Clutterbuck, 1966; Pateman *et. al.*, 1967). Although chlorate toxicity has been varied depending on chlorate concentration, and the nitrogen source used, the mechanism of chlorate sensitivity and toxicity are not clear. One theory is that as a result of NR activity, non (or less toxic) chlorate is converted to the highly reactive toxic substance, chlorite. At variance with this notion, not all mutants which are defective in NR activity are chlorate resistant. Another puzzling aspect is that, it is possible to isolate chlorate resistant mutants which have wild-type NR activity levels.

Furthermore, Cove reported that the presence of chlorate leads to incapability of cells to utilise nitrogen sources normally (as in the absence of chlorate). This suggested that chlorate toxicity is due to chlorate mimicking the effect of nitrate on the control of nitrogen catabolism, without itself (chlorate) being able to serve as a nitrogen source. Additionally, it has been pointed that resistance to chlorate is related to the loss of regulatory ability rather than loss of NR activity (Tomsett and Garrett, 1980; Pelsy *et. al.*, 1991; LaBrie *et. al.*, 1992; reviewed by Cove, 1976 a and b,1979)

In addition to the mutants isolated on the basis of their resistance to chlorate, mutants were also selected by means of the putrescine starvation technique on nitrate as nitrogen source (reviewed by Cove, 1976 a and b, 1979), i.e., were classified on the basis of their ability to utilise certain nitrogen sources. Finally, yet another method of mutant isolation relies on isolating mutants with normal or higher levels of NR activity. This isolation method can be achieved by means of whether the mutant can convert or excrete nitrite. The excreted nitrite can be determined by a pink colour formed after the addition of sulphanilamide as a diazotizing agent and N-(1 naphthyl) ethylene diamine hydrochloride as a coupling agent to the solid medium. Colonies which can convert nitrate to nitrite i.e. have NR activity, stain pink whilst mutants which excrete nitrite exhibits a pink halo (Tomsett and Garrett, 1980)

1.7.2 Molybdate Resistance.

Mutants designated *molA*, were selected for resistance to molybdate (33 mM) with ammonium (10 mM) as the sole nitrogen source. *molA* strains show wild-type growth levels on either nitrate or hypoxanthine as sole nitrogen sources, and wild-type (either induced and uninduced) levels of NR and PH. Such features of *molA* mutants might indicated a possible link between *molA* locus and nitrate metabolism. In addition, *molA* mutants showed sensitivity to increased levels of molybdate (150 mM) when nitrate (10 mM) along with ammonium (10 mM) were used as nitrogen sources. The lack of additional molybdate resistance (ie at 150 mM) on nitrate containing medium suggested that molybdate detoxification mechanism in wild-type is nitrate inducible, whilst in *molA* strains is constitutive . Since, it is an open question whether or not *molA* gene is involved in nitrate assimilation or hypoxanthine catabolism, therefore, it could be involved in the formation of Mo-Co (Arst and Cove, 1970; Arst *et. al.*, 1970; reviewed by Cove, 1979).

An additional class of molybdate resistant designated *molB* mutants, were selected for resistance to molybdate (at 33 mM) with ammonium (10 mM) as the sole nitrogen source. Although *molB* mutants were partially defective in the utilisation of both nitrate and hypoxanthine as sole nitrogen sources, they showed wild-type (induced and uninduced) levels of both NR and PH. Additionally, *molB* mutants showed resistance to increased levels of molybdate

(150 mM) when nitrate (10 mM) along with ammonium (10 mM) were used. The ability of nitrate to protect against molybdate toxicity in the presence of ammonium, indicated that this activity is neither repressed nor inhibited by ammonium. These properties suggests that a molybdate detoxification mechanism might come as a result of the incorporation of molybdate into the NR or another molecule having control properties as NR in order to detoxify molybdate (Arst *et. al.*, 1970; reviewed by Cove, 1979)

It is appropriate to restate here that a relatively high concentration of molybdate (33 mM) can restore the ability of *cnxE* mutants to utilise nitrate and hypoxanthine as the sole nitrogen sources, i.e., the activities of the three molybdoenzymes could be restored to *cnxE* mutants if they are grown in the presence of high concentration of molybdate (see section molybdenum cofactor biosynthesis: Genetics) (Cove *et. al.*, 1964; Arst *et. al.*, 1970; Scazzocchio, 1974; Arst *et. al.*, 1982; reviewed by Cove, 1979).

Resistance to molybdate in certain *A. nidulans* (*galC1*, *galC4*, *gamA66*, *gamB65*, and *gamC66*) strains may be due to uncompleted galactose utilisation, however, loss of activity in galactokinase and/or galactose-1-phosphate uridyl transferase does not lead to molybdate resistance. This resistance comes as a result of loss in phosphatase IV activity, while the hypersensitivity to molybdate in mutants resulted from increased levels of the enzyme. Arst and Cove (1970) concluded

that phosphorylated and carbohydrate-containing compounds play a role in molybdate metabolism, and are possibly involved in the uptake. Where the biosynthesis of such compounds is controlled by either glucose-1-phosphate, uridine diphosphoglucose and uridine diphosphogalactose.

1.7.3 Tungstate Resistance.

It has been shown that tungstate (a molybdate analogue) can be inserted at the molybdenum position into the molybdenum cofactor instead of molybdate (Arst *et. al.*, 1982). A concentration of 33 mM tungstate completely inhibits the growth of *A. nidulans* wild-type with either nitrate or hypoxanthine as sole nitrogen source. However, wild-type cells become five-fold more sensitive to tungstate in the presence than in the absence of either nitrate or hypoxanthine as sole nitrogen source, but less than with nitrate alone on a wide range of nitrogen sources (nitrite, urea, uric acid and glutamate) (Arst, 1968; Arst *et. al.*, 1970; Arst *et. al.*, 1982). It has been demonstrated that tungstate toxicity is competitively reversible by molybdate when used at a concentration two to three times lower than that of tungstate. As discussed before (section 1.5.2 on the molybdenum cofactor biosynthesis) *cnxJ* mutants in presence but not in absence of tungstate fail to grow on nitrate or hypoxanthine as the sole nitrogen source. Since no availabe evidence which indicates that mutations in *cnxJ* would lead to a complete loss of the molybdoenzymes, therefore, such mutations seem to affect only the levels of these enzymes. These results would suggest that *cnxJ* gene might be involved in the

regulation of the cofactor level. Growth inhibition in presence of tungstate was related to competition with molybdate at the uptake level, once inside the cell tungstate instead of molybdate is inserted in the Mo-Co of NR leading to the inactivation of this enzyme (Arst *et. al.*, 1982). Additionally, it has been demonstrated that on nitrogen sources other than nitrate and hypoxanthine molybdate and tungstate toxicity can be competitively reversed by sulphate. Therefore it would seem that sulphate competes with both molybdate and tungstate at the uptake level. Such kind of competitive interaction at the uptake level between the three compounds has been previously described in both prokaryotes and eukaryotes, including *Salmonella typhimurium* (Pardee *et. al.*, 1966), *Chlorella pyrenoidosa* (Vallee and Jeanjean, 1968), *Neurospora crassa* (Ramaiah and Shanmugasundaram, 1962a), and *Aspergillus niger* (Raman *et. al.*, 1962). In *Aspergillus nidulans* it has been reported that mutants defective in sulphate transport (*sB* mutants) showed resistance to molybdate, tungstate, chromate, and selenate (Arst, 1968). Such results tentatively indicated that there is at least one common compound required for the transport of all these structurally similar anions (Yamamoto and Segel, 1966; Tweedie and Segel, 1970).

In higher plants, Labrie and co-workers (1992) reported that the *chl 2* and *chl 6* mutants of *Arabidopsis thaliana* were sensitive to tungstate concentrations that had no effect on wild-type plants. Additionally, these mutants showed tungstate sensitivity, chlorate resistance, reduced levels of NR activity as well as a reduction in the

level of Mo-Co. Therefore it has been suggested that such co-segregated phenotypes might be due to a single mutation (which maps in this case in either *chl l 2* or *chl 6*) (LaBrie *et. al.*, 1992). The *chl 2* mutants although showing low levels of NR activity but, has been thought not to be defective in Mo-Co since, the mutants showed wild-type levels of PH I activity, which has been used as a measurement of the Mo-Co in other organisms (LaBrie *et. al.*, 1992). Also, *chl 2* and *chl 6* mutants show complete sensitivity to tungstate at a concentration of 0.1 mM (about 33 mM was used with the *Aspergillus* strains) and were rescued with 20 μM molybdate. Because tungstate competes with molybdate, the *chl 2* mutants impaired in Mo-Co biosynthesis, might be more sensitive i.e. hypersensitive to tungstate than the wild-type strain (LaBrie *et. al.*, 1992). This result was in broad agreement with the *Aspergillus nidulans* mutants work, using, both *cnxJ* and the cryso-sensitive *cnxC20* . These strains showed more sensitivity to tungstate with nitrate or hypoxanthine as sole nitrogen source than the *Aspergillus nidulans* wild-type, and showed reduced molybdoenzymes levels (Arst *et. al.*, 1982). Finally, the combined activities of the plant mutants (ie. tungsate sensitivity, chlorate resistance, reduced levels of NR but not PHI, as well as reduction in the levels of Mo-Co) lead to the suggestion that these mutants might be Mo-Co defective mutants rather than being regulatory ones, since they either synthesise or utilise or even stabilise the Mo-Co (LaBrie *et. al.*, 1992).

1.7.4 Caesium Sensitivity.

crnA mutants can be easily distinguished from other nitrate assimilation mutants by their ability to grow as the wild-type on nitrate as a sole nitrogen sources (see section 1.3 the transport of nitrate and nitrite, genetics) (Brownlee and Arst, 1983; Unkles *et. al.*, 1991; reviewed by Tomsett, 1991).

When caesium is added to nitrate or nitrite containing minimal medium, caesium inhibits the growth of the mutant *crnA1* completely on nitrate, while on nitrite the growth is reduced rather than completely inhibited, as compared to the wild-type strain which shows resistance to caesium on either nitrate or nitrite. In contrast, growth inhibition with caesium was not observed when other (than nitrate or nitrite) nitrogen sources were used. This lead to the suggestion that there is an additional nitrate uptake system(s) involved which might also transport nitrite (Brownlee and Arst, 1983; Unkles *et. al.*, 1991). Although the mechanisms behind caesium toxicity is unclear, it has been considered that it is related to some kind of interference between caesium and a proposed second nitrate uptake system. In this regard, it has been demonstrated that hypersensitivity shown by mutants to caesium is associated with reduced rates of nitrogen utilisation. Similarly, resistance to caesium was correlated with an increase rate of nitrogen source utilisation, which presumably leads to higher intracellular ammonium, where ammonium protects against caesium toxicity (Arst and Cove, 1973; Arst and Page, 1973; Arst *et. al.*, 1981; reviewed by Cove, 1979).

1.7.5 Regulatory Genes For Nitrate Assimilation .

The nitrate assimilation *crnA-niiA-niaD* genes were found to be under considerable regulation mediated by the products of two unlinked control genes, designated *nirA* and *areA*, in addition to autoregulation (i.e. involved in the regulation of its own synthesis) by *niaD* itself. With regard to the latter phenomena, the kinetic studies results demonstrated that the interruption of NR enzyme synthesis is more likely to be due to a co-repression effect of its own synthesis when it is not in the form of complex with nitrate. That is in addition to the regulatory effect of the *nir* gene, where in the absence of nitrate the NR enzyme bounds with the product of *nir* gene (required for *niaD* expression) and renders it inactive (Cove, 1967; Cove and Pateman, 1969; reviewed by Cove, 1979).

The *areA* Regulatory Gene.

The *areA* gene (maps in linkage group III) product is necessary for the synthesis of many ammonium repressible activities (i.e. nitrogen metabolite repression) mediating the enzymes of nitrate assimilation. Two types of mutant alleles were isolated previously in *areA* gene. The *areA* ^{r}, in which mutants are able to utilise ammonium but not other nitrogen sources, and *areA* ^{d}, which has a growth pattern similar to that of the wild-type i.e., derepressed for some normally ammonium repressible activities. These findings strongly indicated that regulation of expression of the three structural genes in the nitrate assimilation pathway (are subject to nitrogen

metabolite repression) is mediated by the AREA protein, the product of *areA* gene which is the wide-domain regulatory gene that plays a role in mediating ammonium repression (Arst and Cove, 1973; reviewed by Cove, 1979). Recently the *areA* gene has been isolated and sequenced (Kudla *et. al.*, 1990) and the sequence analysis indicate the presence of single ORF that encodes a polypeptide of 719 amino acids. This ORF shows strong homology with the equivelent regulatory gene from *N. crassa nit-2* (Fu and Marzluf, 1990 a; Kudla *et. al.*, 1990). Sequence analysis indicate also that the essential region of the gene consists only of 223 amino acids. This region was found to contain domains that could be involved in interactions with transcription factors such as an activator domain, DNA-binding domain or may be nuclear localisation signal. Analysis of the derived amino acid sequence of this essential region revealed an acidic region which is responsible for transcriptional activation of nitrogen regulated genes it controls (Kudla *et. al.*, 1990), the second domain within this essential region is a putative zinc finger which is bound by two pairs of cysteine. This has been shown to have a regulatory role. The zinc finger domain showed approximately 73% identity with the putative zinc finger domain of the yeast *S. cerevisiae* regulatory gene product GLN-3 (Minehart and Magasanik, 1991) and the most near perfect identity with the finger domain in the *nit-2* product of *N. crassa* (Fu and Marzluf, 1990 a).

The *nirA* Regulatory Gene.

nirA mutants are unable to utilise either nitrate or nitrite as sole nitrogen source. Although, the growth pattern is similar to *niiA* mutants, they can be easily distinguished by the nitrite excretion test, since *niiA* mutants excrete high levels of nitrite into the medium whereas, *nirA* mutants excrete little or no nitrite. Biochemical studies indicated that, *nirA* mutant alleles either lead to non inducible (*nirA* $^{-}$) or constitutive (*nirA* ^{c}) synthesis of both NR and NiR. The obtained results from the two contrasting types of mutant alleles in the same gene strongly suggest that the positively acting pathway-specific control protein NIRA, encoded by the *nirA* gene is required for nitrate induction of the nitrate assimilation pathway enzymes (Pateman and Cove, 1967; Cove, 1976 a; reviewed by Cove, 1979). Recently, the *nirA* gene has been isolated and sequenced (Burger *et.al.*, 1991 a, b). The NIRA protein which consists of 892 amino acids was found to contain a putative zinc finger (i.e. zn(II)2 Cys6 binuclear cluster) DNA-binding domain near the N-terminal end of the protein. In addition, the protein possessed an acidic region and two proline rich regions as well as a basic region. The exact function of these regions is unknown, but it has been suggested that these domains might be involved in gene activation, whilst the latter still of unknown function. In *Neurospora crassa* the equivalent pathway-specific regulatory genes necessary for nitrate induction, designated *nit-4* (Yuan *et. al.*, 1991), has also been sequenced. Comparisons of the deduced amino acid sequence indicate that there is no similarity between the two deduced proteins at the carboxyl terminus.

However, 90% of residues were found identical within the zinc finger domain. Furthermore, another region of similarity (approxi 81%) of unknown function was found distal to the zinc finger and the acidic domains, The suggested function of this distal region is that it may be involved in the recognition of the inducer.

Other Genes Which Might Have A Regulatory Rule.

A structural gene for nitrate uptakes *crnA*, is also subject to repression by ammonium and induction by nitrate. The induction is mediated by the *nirA* gene product (Unkles *et. al.*, 1991). The *nirA* gene maps in linkage group VIII (positive-acting regulatory gene, mediating nitrate induction). Brownlee and Arst (1983) concluded that the expression of *crnA* is independent of *nirA* gene, which controls the activities of both enzymes in the pathway (NR and NiR). The *areA* (positive-acting regulatory gene, mediating nitrogen metabolite repression) gene product is necessary for the regulation of all three gene expression (Unkles *et. al.*, 1991; reviewed by Cove,1979). DNA sequencing has shown that *nirA* is indeed a regulatory gene. It has been reported that the nitrogen regulatory circuit was shown to be influenced directly or indirectly by a number of other identified genes including, *tamA* gene (Kinghorn and Pateman, 1975), *meaB* gene (Arst and Cove, 1969; Arst and Page, 1973), and *gdhA* gene (Kinghorn and Pateman, 1973).

1.8 Fungal Transformation.

Genetic transformation may be defined as the transfer of heritable traits by introduction of naked DNA molecules into cells. Also, it may be defined as a genetic process through which the transfer of genetic information can occur (Mishra, 1985). Operationally transformation provides a method for self-cloning of fungal genes by complementation of mutant phenotypes (Johnson, 1985). This self-cloning approach is an attractive cloning method, particularly for *A. nidulans* and *N. crassa* which have many well characterised mutant alleles. The self-cloning technique is based on the usage of fungal genomic libraries constructed in plasmid or cosmid vectors to complement fungal strains after transformation. A complementary clone carrying the gene of interest can then be isolated by either rescuing in the bacterium *E. coli* (marker rescue) or by subcloning from positive pools of clones from plasmid or cosmid genomic libraries (Gems *et. al.*, 1991; Gems *et. al.*, 1994; reviewed recently by Riach and Kinghorn, 1996).

Certain model organisms such as *A. nidulans* have a well characterised genetic linkage map (Figure 1.3), physical chromosome characterisation (physical or contiguous maps) and chromosome specific gene libraries constructed in plasmid, cosmid and phage vectors, and a number of already cloned genes. A more laborious procedure than self-cloning which takes advantages of these features is 'chromosome walking' : the isolation of overlapping cloned fragments, so as to proceed from a given point to any gene of interest.

Clones containing overlapping DNA fragments located progressively distal to the isolated gene are tested for phenotypic complementation of a mutation in the desired gene using standard transformation protocols. Overlapping clones can be organised into contiguous chromosomal regions (termed contigs) and used to construct physical genomic maps (contig maps). In *A. nidulans*, cosmid libraries are available for each chromosome (Gibson *et. al.*, 1987; Wahl *et. al.*, 1987; Brody *et. al.*, 1991).

A recent transformation protocol is the method of transforming protoplasts by electroporation (the use of high voltage electric pulse to allow reversible permeabilisation of the cell membrane and uptake of DNA. The most common method for fungi transformation involves the preparation of protoplasts in the presence of an osmotic stabiliser (high salt or sugar concentration) in order to prevent cell lysis, followed by their successful regeneration (transformants grown on osmotically buffered selective medium) (see Figure 2.1, cloning strategy). Such transformation technology provides many ways of altering the genetic characteristics of fungi, and has been used for studying mechanisms that control growth, metabolism and development. Also, for the isolation and manipulation of genes of potential importance, ie. in medicine (genes required for antibiotics production), in industry (genes required for production of food additives such as citric acid).(Timberlake and Marshall, 1989; reviewed by Riach and Kinghorn, 1996).

Although *A. nidulans* itself, is not of direct or obvious commercial importance its properties makes it an attractive and useful model organism suitable for studying metabolic and developmental regulation. Also, it might act as a host for expression of genes cloned from other species e.g. as a host for expression of cloned genes from higher eukaryotes (Timberlake, 1980, Tilburn *et. al.*, 1983; Arst, 1981, 1983; Johnstone, 1985).

Requirements For Transformation.

Three major requirements are needed for a successful transformation experiment, these are listed below:

1- A vector carrying a selectable marker, which after entry into the cell results in selective growth of only transformed cells.

2- Entrance of DNA into the cell and successful regeneration of the treated cells. This success depends on the viability of young protoplasts with competent cell membrane able to attach incoming DNA (ie. the vector) by rendering the cell wall permeable to the DNA. Selective conditions are subsequently applied to detect only the transformants that have incorporated and expressed the incoming DNA, and are capable to grow under selective conditions.

3- The expression and stable maintenance of transformed genetic material. Such maintenance can be achieved either by the autonomous replication, or by integration into the host chromosome (Johnstone, 1985; Gems *et. al.*, 1991; reviewed by Riach and Kinghorn, 1996).

Factors Affecting Transformation Frequency.

The frequency of transformation in fungi is dependent on several factors listed below:

1. The nature of the recipient strain used in transformation.
2. The physiological state of the protoplasts, in which the preparation of viable transformable protoplasts depends on the age of cells, the choice of enzyme used for cell wall digestion, also the optimal timing for enzymatic digestion is crucial.
3. The nature of donor DNA and its purity.
4. The shape and size of the transforming DNA.
5. The ratio of the transforming DNA to that of the donor one.
6. Regeneration conditions. Also the ratio between the DNA and the protoplasts, and the density at which the protoplasts are plated on the selective medium after the treatment with the DNA is important (Mishra, 1985; reviewed by Saunders *et. al.*, 1986; Fincham, 1989).

General Application Of Transformation.

Fungal transformation is a technique with applied and/or practical purposes:

1. The isolation of certain DNA sequence from a mixture of DNA molecules in the fungal cell (as discussed above).
2. Insertion of an artificially synthesised nucleotide sequence, in order to examine its expression in fungal cells.
3. Modification of a desired nucleotide sequence in order to examine its effect on the expression of a gene.

4. For the commercial production of enzymes and proteins especially with elevated levels through the construction of hybrid plasmids. In this regard the gene that encode a desired enzyme or protein is linked to a fungal promoter and transcriptional activator carrying a signal sequence that finally terminates sequence and enables the cell to secrete the required product to the outside medium.
5. In biotechnology, e.g. for food industry, ethanol and organic acid fermentation's, plant pathology, disposal of plant waste
6. For gene disruption perposes (reverse genetics) (Mishra, 1985; Fincham, 1989; Peberdy, 1991).

Molecular Cloning Techniques.

The DNA is normally partially digested with a restriction endonuclease into relatively small fragments (10 to 45 kb), is then inserted into a vector (plasmid, bacteriophage, or cosmid) carrying selectable marker (drug resistance) and cohesive ends which facilitates the ligation. Such hybrid plasmid vector may be used for transforming the host (e.g. *E. coli* , *Aspergillus* and yeast), where the presence of recombinant plasmid may be detected through the selectable marker. The presence of certain gene in this recombinant vector can be identified by a number of techniques, such as the complementation of mutants, immunological screening or hybridisation with a specific probe which could be DNA RNA or cDNA (reviewed by Mishra, 1985).

Vectors Used In *Aspergillus.*

The choice of vector for molecular cloning depends on both its capability of isolating and transferring the cloned gene via *E. coli*, and on the nature of the gene fusion. Additionally, a vector should have a selectable marker and a unique cloning site, also it must be able to replicate in both fungus and bacteria. A substantial efforts have been put to develop a kind of vector from filamentous fungi which could replicate autonomously. The advantages of this type of vector not only they enhance the transformation frequencies but also they may be recoverd easily in *E. coli.* Recently a vector (and derivatives) have been developed in *A. nidulans* in which DNA insert (designated AMA1) which replicates autonomously with increased transformation frequency. Different kinds of vectors based on this have been used for gene cloning in fungi, their main characteristics are illustrated below (under plasmids section) (reviewed by Mishra, 1985; Saunders *et. al.*, 1986; Riach and Kinghorn, 1996).

Features Of Plasmids Used In This Study.

These vectors were developed because of their small size, easy to replicate and possessed a defined number of cleavage sites for common restriction endonucleases. The most useful (i.e. transforming at high frequency) *A. nidulans* replicating plasmids are those designated ARp1, pDHG25, and pHelp, pDHG25 and pHelp, can be linearised at a unique site, thus directing the insertion of DNA fragment (Figure 1.5). **ARp1:** The replicating plasmid (*Aspergillus*

replicating plasmid), consists of pILJ16 with a 6.1 kb insert (ie. making total of 11.5 kb) (Johnstone *et. al.*, 1985; Gems *et al.*, 1991). The insert (AMA1: autonomously maintained in *Aspergillus*) sequences came from *A. nidulans* . This vector exists in more than one copy in transformant cells. This DNA insert (i.e. AMA1)is responsible for autonomous replication of the plasmid vector, and has been used successfully for both increasing the transformation frequency and for the synthesis of 'instant gene banks' when cotransformed with genomic DNA into an organism.. This vector is an autonomously replicating plasmid which transforms *A. nidulans* at frequencies much higher (up to 250 times) than pILJ16 (the parental plasmid). **pDHG25:** an autonomously replicating plasmid which is slightly smaller than ARp1 (10.5 kb), and the outer 0.5 kb of AMA1 on a *Hin* dIII fragment, also it has a unique *Bam* HI site which directs the insert to this site. It transforms at frequencies up to 1/40 of that of ARp1, but still higher (50 times) than the parental plasmid pILJ16. **pHelp:** Markerless ARp1 derivative (8 kb in size), having a unique *Bam* HI site, in contrast, with the other two plasmids it does not carry the *argB* selective marker, but it has the AMA1 sequence, which enhances the transformation efficiency. (Gems *et. al.*, 1991; Gems *et. al.*, 1993; reviewed by Mishra, 1985; Martinelli and Kinghorn, 1994; Riach and Kinghorn, 1996).

1.9 Objectives Of This Study.

The overall aim of this project is to identify, isolate and characterise genes involved in nitrate assimilation. The first specific

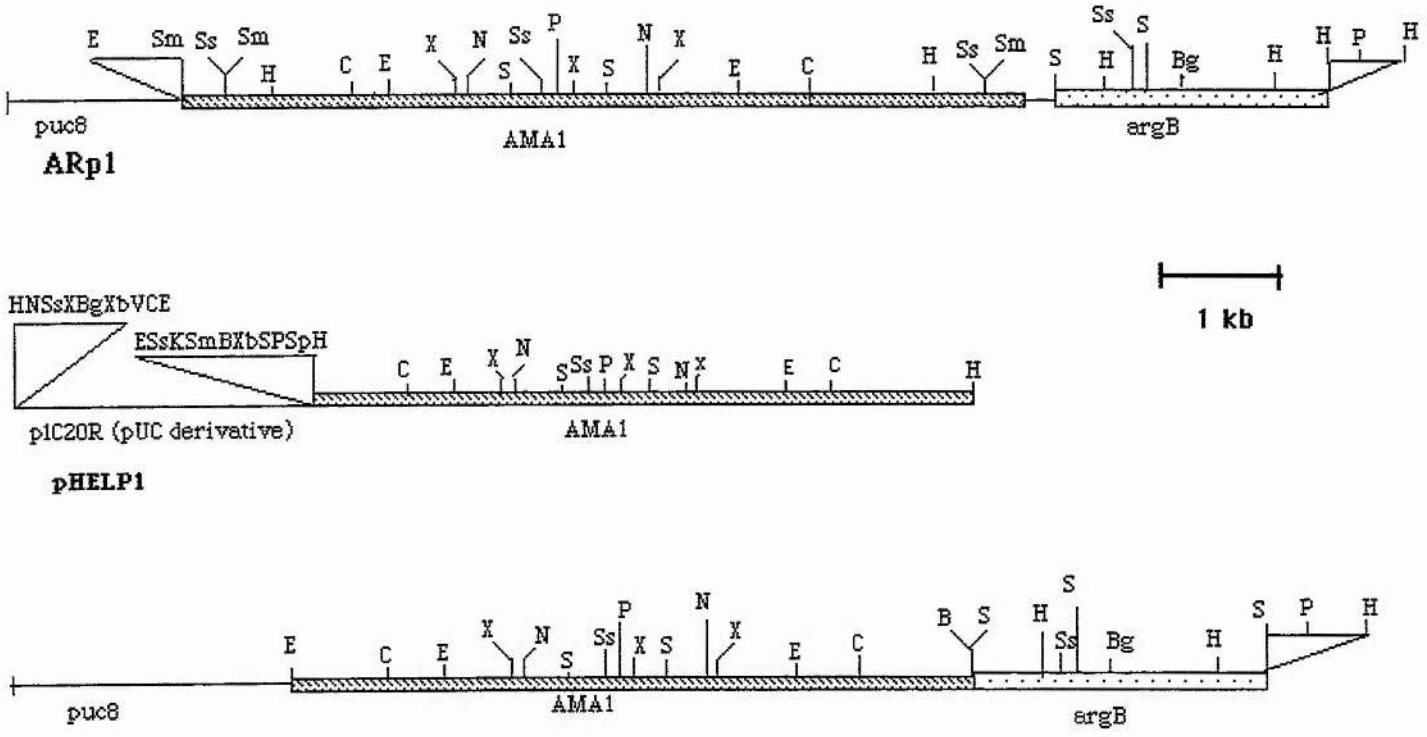


Figure 1.5. The Autonomously Replicating Plasmids, Which Contain The *Aspergillus nidulans* AMA1 Sequence, That Increases The Transformation Effeciency. E = *EcoR* I; Sm = *Sma* I; Ss = *Sst* I; H = *Hind* III; C = *Cla* I; X = *Xho* I; N = *Nru* I; S = *Sal* I; P = *Pst* I; Bg = *Bgl* II; Xb = *Xba* I; K = *Kpn* I; V = *EcoR* V; B = *Bam* HI-unique site in pDHG25 and pHELP 1, no *Bam* HI in ARp1

aim is to study and identify genes involved in nitrate and nitrite transport in *Aspergillus. nidulans*. The second objective is to isolate and characterise, at the molecular level, the genes required for the synthesis of the molybdenum cofactor from both *Aspergillus nidulans* and the higher plant *Arabidopsis thaliana*.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Organisms And Strains Used In This Research.

2.1.1 *Aspergillus. nidulans.*

Strains Used For Transformation Experiments.

Strain Number	Genotype
GH5.4	*yA2 methH2 argB2 biA1 cnxH4*
G063	*biA*1 *cnxH* 4
G338	*pantoC3 Sc12 cnxH3*
∝ 56	*biA1 cnxH3*

Strains Used For Mutagenesis.

Strain Number	Genotype
G1	*biA1*
A220	*yA2 pyroA4*
SAA1040	*yA2, methH2, niaD53, argB*$^{+}$ [*gdhA-niaD*] {transformant No: 9-6}.
SAA1032	*yA2 wA3, meth H2, niaD4, argB*$^{+}$ [*gdhA niaD*]{transformant No: 12-4}.

SAA1023a	*yA2, wA1, methH2, niaD18, arg B*$^+$ [*gdhA-niaD*] {transformant No: 12-5}.
SAA1023b	*yA2, wA1, methH2, niaD18, arg B*$^+$ [*gdhB-niaD*] {transformant No: 20}.

Mutants Used For Enzyme Assays.

Temperature-sensitive *cnx* and various non-conditional *crn* mutants isolated in this study are listed below. In addition *gdhA* and *gdhB* transformants (see section strains used for mutagenesis), were included.

Strain Number	Genotype
G1	*biA1*
N1458	*yA2 biA1 crnA1*
GH688	*biA1 crnA688*
GH976	*biA1 crnB976*
GH967	*biA1 crnB967*
GH561	*yA2 pyroA4 crnC561*
GH1056	*biA1 crnC1056*
GH1116	*biA1 crnD11116*
GH140	*yA2 wA1 methH2 cnxA140* ts
GH142	*yA2 wA1 methH2 cnxF142* ts
GH251	*yA2 methH2 cnxH251* ts
GH255	*yA2 methH2 cnxH255* ts
GH261	*yA2 methH2 cnxH261* ts

SAA1040.	*YA2, methH2, niaD53, argB*$^{+}$ [*gdhA-niaD*] {transformant No: 9-6}.
SAA1032	*YA3, wA3, meth H2, niaD4, argB*$^{+}$ [*gdhA-niaD*] {transformant No: 12-4}.
SAA1023	*YA2, wA1, methH2, niaD18, arg B*$^{+}$ [*gdhA-niaD*] {transformant No: 12-5}.
SAA1023	*YA2, wA1, methH2, niaD18, arg B*$^{+}$ [*gdhB-niaD*] {transformant No: 20}.

Strains Used For Nitrate Uptake Assays.

Strain Number	Genotype
G1	*biA1*
N1458	*yA2 pyroA4*
GH688	*biA1 crnA688*
GH946	*biA1 crnA946*
GH300	*biA1 crnA300* ^{ts}
GH538	*biA1 crnA538* ts
GH496	*yA2 pyroA4 crnA496* ts
GH976	*biA1 crnB976*
GH967	*biA1 crnB967*
GH974	*biA1 crnB974* ^{ts}
GH1030	*biA1 crnB1030* ^{ts}
GH561	*yA2 pyroA4 crnC561*
GH1056	*biA1 crnC1056*

GH635	*yA2 pyroA4 crnC635* ^{ts}
GH640	*yA2 pyroA4 crnC640* ^{ts}
GH1116	*biA1 crnD1116*

Strains Used For Nitrite Uptake Assays.

Wild-type,*crnA1*, *meaB6*, *niiC628*, and *tamA105*, mutant strains carrying markers in standard use, which have been described previously (Arst and Cove, 1969; Cove, 1979; Clutterbuck, 1974; Kinghorn and Pateman, 1975). Other *crn* mutants were isolated during this study by spontaeous, NTG or DEO mutagenesis of the wild-type.

Strain Number	Genotype
G1	*biA1*
N1458	*yA2 biA1 crnA1*
GH967	*biA1 crnB967*
GH1056	*biA1 crnC1056*
GH1116	*biA1 crnD1116*
G326	*biA1 meaB6*
DC	*pyroA4 niiC628*
JK 100	*paba pyroA4 tamA105*

Strains Used For DNA Sequencing.

Strain Number	Genotype
GH427	*biA1 cnxH427*
GH911	*biA1 cnxH911*
GH261	*yA2 methH2 cnxH261* ts
N1458	*yA2 biA1 crnA1*
GH1009	*biA1 crnA1009*
GH1087	*biA1 crnA1087*
GH517	*yA2 pyroA4 crnA517*
GH688	*biA1 crnA688*
GH760	*biA1 crnA760*
GH775	*biA1 crnA775*
GH1025	*biA1 crnA1025*

2.1.2 *Esherichia. coli*

The DH5 ∝ strain was used for the propagation of all plasmids.

The DH1 strain was used for the propagation of cosmid clones.

Strain Number	Genotype
DH5 ∝	(F^-, *end* A1, *hsd* R17, *sup* E44, *thi* -1, *rec* A1, *gyr* A96, *rel* A1, F80 ^{R} , *lacz* Δ M15).
DH1	(F^-, *rec* A1, *end* A1, *gyr* A1, *hsd* R17, *sup* E44).

2.1.3 *Arabidopsis*

Arabidopsis. thaliana

2.2 Growth Media.

2.2.1 *A. nidulans* Growth Media.

All solutions and media were prepared in distilled water. These were sterilised by autoclaving at a pressure of 15 pounds per square inch, at 121°C temperature, for 20 min, unless otherwise specified. All stock solutions required for media preparation (i.e. salt solutions, vitamin solution, trace-elements solution, and supplements) in addition, to the complete media were based on that described previously by Cove (1966). Minimal media was based on that described by Pontecorvo *et. al.*, (1953) as modified by Roberts (1963).

Salt Solution.

Components	g/L
KCl	62
$MgSO_4$	26
KH_2pO_4	76

Vitamin Solution.

Components	g/L.
Biotin	2.5
Nicotinic acid	2.5
PABA	0.8
Pyridoxine hydrochloride	1.0
Pantothenate	2.0
Riboflavin	2.5
Aneurin	1.5
Choline hydrochloride	20.0

Trace Element Solution.

Components.	g/L.
$Mo_7O_{24}.4H_2O$	1.1
H_3BO_3	11.1
$CoCl_2.6H_2O$	1.6
$CuSO_4.5H_2O$	1.6
EDTA	50.0
$FeSO_4.7H_2O$	5.0
$MnCl_2.4H_2O$	5.0
$ZnSO_4.7H_2O$	22.0

The solution was boiled with continuous stirring, until complete

dissolving, and the solution was allowed to cool to 60°C. The pH was adjusted to 6.5 with 5 M NaOH, and the total volume was made up to 1 litre with sterile distilled water. After autoclaving at a pressure of 15 pounds per square inch, 121°C, for 20 min, the solution was stored at 4°C in a dark bottle.

Minimal Medium.

Components.	g/L.
Salt solution	50.0 ml.
Vitamin solution	1.0 ml
Trace-elements solution	1.0 ml
Glucose	10.0 g.
Agar	12.0 g.

The pH was adjusted to 6.5 with 5 M NaOH. The vitamin solution or any other required supplement, or nitrogen source was added at a standard level, to molten agar media at 50°C.

Complete Medium.

Components.	g/L
Salt solution	50.0 ml
Vitamin solution	1.0 ml
Trace-elements solution	1.0 ml

Glucose	10.0 g.
Peptone	2.0 g.
Yeast extract	1.0 g.
Casein hydrolysate	1.0 g.
Agar	12.0 g.

The pH was adjusted to 6.5 with 5 M NaOH. Liquid culture media whether minimal or complete, have the same constituents as solid media except that liquid media lacking the solidifying agent (ie. agar). Before inoculation of the culture conidiospores were suspended in physiological saline / Tween 80 solution (the latter being a wetting agent to prevent the spores and mycelia from clumping and adhering to the sides of the flask).

2.2.2 *Esherichia. coli* Growth Media.

Luria Agar Medium.

Components	g/L.
---	---
NaCl	10.0 g.
Tryptone	10.0 g.
Yeast extract	5.0 g.
Agar	12.0 g.

The pH was adjusted to 7.5 with 5 M NaOH. After autoclaving at a pressure of 15 pounds per square inch, 121°C, for 20 min, the media was allowed to cool down to 50°C before pouring into Petri dishes.

Luria Agar Base Miller Dehydrated.

30.5 g were suspended in 1 litre of distilled water, the pH was 7.0.The suspension was allowed to boil in order to dissolve completely. The media was sterilised by autoclaving at a pressure of 15 pounds per square inch, 121°C, for 20 min and allowed to cool down to 50°C before pouring in plates.

Luria Broth Base (Miller's LB Broth Base).

25 g were dissolved in 1 litre of distilled water, the pH was 7.0. The media was sterilised by autoclaving at a pressure of 15 pounds per square inch, 121°C, for 20 min, and allowed to cool down to 50°C before pouring into plates or adding before the required antibiotic.

Antibiotics.

Ampicillin.

0.5 g was dissolved in 40 ml distilled water / 10 ml Tris-HCl pH 7.5, (filter sterilised). 10 ml of stock were used per litre and that gave a final concentration of 100 μg/ml.

Kanamycin.

0.5 g was dissolved in 40 ml distilled water and 10 ml Tris-HCl pH 7.5, (filter sterilised). 5 ml of stock were used per litre and that gave a final concentration of 50 μg/ml.

2.2.3 *Arabidopsis thaliana* Growth Medium.

A day before planting *Arabidopsis thaliana* seeds, several trays of peatmoss soil were prepared and irrigated well inside the greenhouse. *Arabidopsis thaliana* seeds were mixed with two thirds of fine soil particles and spreaded on the surface of the soil in the trays, covered by a thin layer of dried peatmoss. Trays were irrigated once every 4 days (seeds usually started germinating after 10 days of planting). After maturation (stage of flowering) plant leaves were collected one by one, and sterilised in 70% ethanol for 15 min, washed with sterile water three times each of 10 min duration. Sterile leaves were weighed into small amounts (0.5 g), and placed in alaminium foil. The leaves were placed in liquid nitrogen for 30 min before transferring to -70°C storage.

2.3 Specific Methodology.

2.3.1 *Aspergillus nidulans.*

2.3.1.1 Growth And Storage Of Strains.

Generally, plates were inoculated by single inoculum (i.e. a point inoculation) from pure cultures using a sterile needle in an upside down position in order to avoid scattering conidia over the plate surface, as well to avoid contaminants. Cultures were grown at 37°C unless otherwise specified. Growth tests were carried out by incubation at different temperatures (normally 25°C, 30°C, 37°C, and 42°C was used). Cultures of temperature-sensitive *cnx* mutants

needed for nitrate reductase assays were grown at 25°C. Cultures required for nitrate and nitrite uptake studies were grown at either 25°C or 37°C, or at both temperatures. Stock cultures of all strains were grown at the required temperature on agar slobes of complete media, and stored in the cold room or at 4°C.

2.3.1.2 Preparation Of Suspensions Of Conidiospores.

Petri dishes were flooded with 20 ml of sterile saline-Tween 80 solution in the fumcupboard. By using a sterile spatula, conidia were collected carefully from the surface of the plate by scraping the mycelia. The suspension was transferred to 30 ml sterilin tubes and vortexed well before inoculation.

2.3.1.3 Large-Scale Fungal Genomic DNA Preparation (Using The Nucleon Kit, ScotLab, U.K)

a) 0.3 g pressed wet weight mycelium were ground to a fine powder in liquid nitrogen (a roughly equivalent amount of a freeze-dried mycelium can also be used).

b) The powder was suspended in 2 ml Nucleon reagent b in a 15 ml screw- capped polypropylene tube with 15 mm internal diameter .

c) 10 mg/ml RNase were added and incubated at 37°C for 30 min.

d) 1.5 ml of 5 M Na perchlorate were added and mixed, kept at room temperature for 15 min in a orbital shaker (at a speed of approx. 100 rpm).

e) The mixture was incubated on a receprical shaking water bath at 65°C for 25 min.

f) 5.5 ml chloroform (previously stored at -20°C) was added and the solution was shaken at room temperature for 10 min.

g) The tube was centrifuged at 800 x g (2200 rpm) in a bentch top centrifuge (MSE, Mistral 1000) for 4 min.

h) 800 ml Nucleon silica suspension was added (shaken vigorously to resuspend) without re-mixing and centrifuged at 1400 x g (2950 rpm) for 5 min.

i) The upper layer was transferred to a further tube, and 1 vol of 100% ethanol (previously stored at -20°C) was added.

j) The tube was gently inverted until a thread like DNA precipitate could be spooled out using a sterile pasteur pipette.

k) The DNA was washed with 70% ethanol by swirling the pipette, transferred to a new tube , dried and suspended in 100 ml TE solution.

l) The DNA was kept at 4 °C overnight, to dissolve completely.

m) On the following day the DNA was incubated at 50°C for10 min, if the DNA had not dissolved completely.

n) DNA concentration was determined by running 1 μl on 0.8% agarose gel.

2.3.1.4 *Aspergillus. nidulans* Transformation.

Solutions used.

1 X STC (sorbitol Tris calcium solution).

1.2 M sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$.

2 X STC.

2.4 M sorbitol, 20 mM Tris-HCl pH 7.5, 20 mM $CaCl_2$.

Trapping buffer.

0.6 M sorbitol, 100 mM Tris-HCl pH 7.0

Osmotic medium.

1.2 M $MgSO_4$, 10 mM NaOP pH 7.0 (i.e 57.7 ml of 1 M Na_2HpO_4 plus 42.3 ml of 1 M NaH_2pO_4 both at pH 7.0, continue to 1 L with distilled water to 0.1 M sodium phosphate buffer, after mixing 10 mM of this buffer with 1.2 M $MgSO_4$ adjust the pH to 5.8 with 0.2 M Na_2HpO_4).

NB: The transformation method used was based on that reported by Tilburn *et. al.*, (1983).

4- Days Before Transformation.

Complete plates (Cove, 1966) were inoculated in a wheel pattern with an inoculum from a pure culture, incubated at 37°C.

The Day Before Transformation.

a) 10 ml saline Tween 80 solution were added to the pure plate, and by blent sterile glass rod the conidia were collected and transferred to sterile tube and vortexed.

b) 400 ml minimal liquid culture (10 mM ammonium and the required suppliments of the strain were added), were inoculated and kept frozen at 20°C, at 8 pm. The culture was incubated at 25°C, 250 rpm for 13 h (ie. only very young cells were used).

c) The media, solutions, materials (all in sterile form) requirements for the experiment were made up ie. stocks of supplements, solid minimal media +1.2 M sorbitol , Corex tubes, spatula, mira-cloth, sterile distilled water, refrigerated 0.6 M $MgSO_4$, 200 ml conical flasks, 1 X STC, 2 X STC solutions, trapping buffer, and osmotic media.

The Transformation Day.

a) The following sterile solutions were pre-cooled in an ice bucket. 1 X STC, 2 X STC, trapping buffer, conical flask, Corex tubes, 60% PEG, osmotic media

b) Preparation of enzymes precooled in ice. 2 ml solutions of 10-20 mg/ml osmotic medium of the commercial enzyme preparation of complex mixture of several hydrolytic enzymes, Novozyme 234 [batch number 1961 or 1906]. Also 1.5 ml BSA solution of 12 mg /ml in osmotic medium .

c) Protoplast (spheroplasts) preparation. The mycelium was harvested through sterile mira-cloth, and washed with sterile refrigerated 500 ml of 0.6 M $MgCl_2.7H_2O$, and resuspended in a sterile precoold conical flask containing 5 ml cold osmotic medium, and incubated on ice. After vortexing, Novozyme 234 batch number 1961 or 1906 solution was added to the cells, and incubated in ice for 5 min. The BSA solution was added and the mixture was incubated in a receprical shaking water bath at 25°C for 2.5 h at 60 rpm/min. Formation of protoplasts was monitered microscopically (Compound microscope, CARL Zeiss Jena, East Germany) , after the incubation

period, the mixture in the flask was divided into two sterile Corex tubes, very gently. 4 ml of osmotic medium were added to each tube, followed by an overlay of an equal vol of cold trapping buffer, very slowly on the side of the tube in order not to mix the contents. After balancing the tubes the protoplasts were separated from the mycelial debris by centrifugation at 4°C and 5000 rpm (Sorval RC5C, HB-4 rotor) for 20 min. The mycelial debris formed a pellet at the bottom of the tube, whilest the protoplasts floated forming a bushy band at the interface, By using a sterile pasteur pipette the entire band of protoplasts was transfered into two sterile Corex tubes on ice, and equal vol of cold 1 X STC was added to each tube followed by centrifugation at 7000 rpm, 4°C for 10 min. The protoplasts were suspended in just sufficient vol of 1 X STC (depending on the number of different DNA preparation used).

Preparation Of DNA.

The cloning strategy for the isolation of *cnx* genes is shown in Figure 2.1. DNA preparation can be made during the incubation period of protoplasts with the Novozyme. In each transformation experiment approximately 5 μg *Sau*3A1 partially digested genomic DNA with 20 μg plasmid cutted with *Bam*H1 were used (for details see results section), equal volume of 2 X STC was added to the DNA solution and the total volume was continued with 1 X STC up to 50 μl

DNA Entry.

50 μl of the protoplasts were transferred to the DNA tube, 25 μl of 60% ice-cold PEG were added to the mixture, which was incubated on ice for 30 min. 1 ml of the 60% PEG was added to each tube, and the tubes were rotated very gently for a few minutes, incubated at room temperature for 30 min. 5 ml of 1 X STC were added to each tube, the mixture was centrifuged at 4500 rpm for 10 min. Finally the pellet was suspended very gently in 300 μl of 1 X STC solution.

Plating out.

Control system.

Already prepared solid minimal + 1.2 M sorbitol medium was melted in a microwave and placed in a water bath at 50°C. The required supplements and 10 mM ammonium (except the control tube where 10 mM nitrate were added) were added and the medium was poured into sterile plastic petri dishes. At the same time the following eppendorf tube solutions were prepared.

a) Tube H_2 contains: 990 μl sterile H_2O + 10 μl protoplasts.

b) Tube S2 contains: 990 μl sterile 1 X STC + 10 μl protoplasts.

c) Tube S3 contains: 900 μl sterile 1 X STC + 100 μl from S2 tube.

d) Tube S4 contains: 900 μl sterile 1 X STC + 100 μl from S3 tube.

e) Tube S5 contains: 900 μl sterile 1 X STC + 100 μl from S4 tube.

f) Tube S6 contains: 900 μl sterile 1 X STC + 100 μl from S5 tube.

g) control tube contains: 990 μl sterile 1 X STC + 10 μl protoplasts.

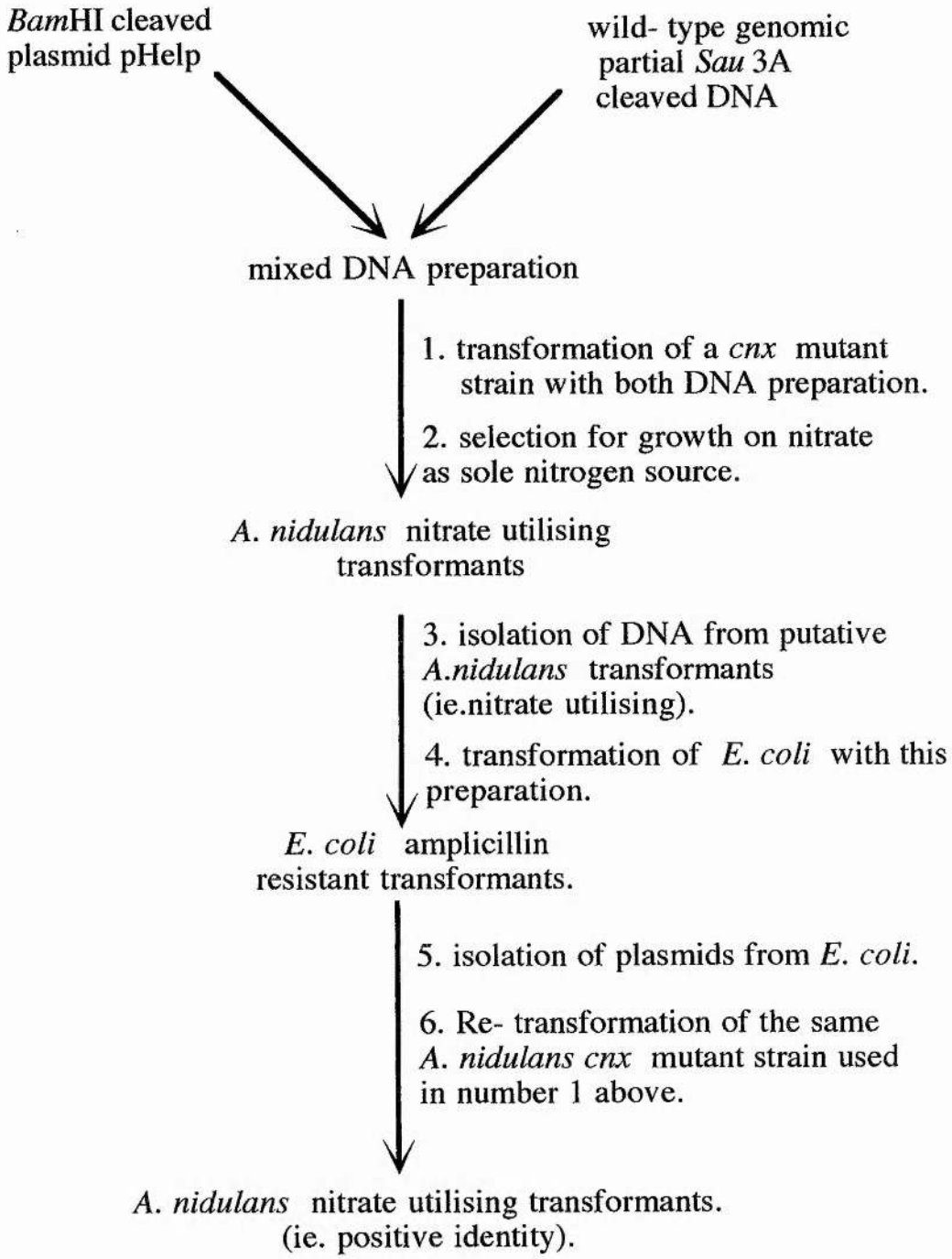


Figure 2.1. The Cloning Strategy For The Isolation Of The *cnx* Genes. Rearrangements most likely occur during pHELP cloning, but rescued plasmid can be used as probe to isolate genomic and cDNA clones. pHELP is an autonomously replicating plasmid, which is 8 Kb in size, has a unique *Bam* HI site, has the AMAI sequence (which shows

100 μl from each microfuge tube (H2 through to control tube as above) were spreaded using a sterile blend glass rod, over medium. The plates were incubated at 37°C for at least two days.

Test System.

300 ml solid minimal +1.2 M sorbitol medium was melted as before and incubated at 50°C. The required supplements were added together with 10 mM nitrate as the sole source of nitrogen for selection, after that the medium was poured into sterile Petri dishes. The mixtures of protoplasts and DNA were spreaded using a sterile blend glass rod over medium in plates. The plates were incubated at 37°C for 5 days.

2.3.1.5 Fungal DNA Mini- Preparation.

The method used was that according to Leach *et. al.*, (1986).

LETS Buffer.

0.1 M LiCl, 10 mM EDTA, 10 mM Tris- HCl pH 8.0,
0.5% SDS.

DNA Preparation.

a) 10 ml of liquid minimal medium (plus supplements of the strain and nitrate 10 mM) were inoculated with a loopful of the transformant's conidia, and incubated for 16-30 h at 37°C at 200 rpm.

b) The mycelium was harvested through Mira-cloth, washed with sterile distilled water, and lyophilised for 24 h.

c) The mycelium was transfered to a microfuge tube and mechanically diced, 0.7 ml LETS buffer was added. Which was then covered with Nescofilm and vortexed at maximum speed (14000 rpm) for 2 min.

d) 1 ml phenol: chloroform: isoamylalcohol (25:24:1) solution was added, vortexed at medium speed for 30 sec.

e) The tube was centrifuged at 3000 rpm for 5 min.

f) 500 ml of the clean aqueous phase were transfered to a sterile Eppendorf tube. 1 ml of 100% ethanol was then added, shaked gently, and incubated at -70°C for 15 min.

g) The tube was centrifuged for 15 min at 14000 rpm.

h) The pellet was dried and resuspended in 40 μl TE solution, 10 mg/ml RNase were added and incubated at 37°C for 3 h.

2.3.1.6 The Generation Of Chlorate Resistant Mutants *Aspergillus nidulans* Nitrate defective, By Spontaneous And Induced mutagenesis.

2.3.1.6.1 Spontaneous Mutations.

Mutants resistant to chlorate, were selected by adding the conidial suspension from each strain separately, to a molten glucose supplemented minimal medium. The media contained, a vitamins solution, sodium chlorate (300 mM), sodium deoxycholate (0.08%) (which restricts colony size), and a sole nitrogen source. Three different nitrogen-sources, were used in these experiments and as these together with the concentrations used follows: proline (10 mM),

glutamate (10 mM) and uric acid (2, 6, 8- Trioxypurine) (10 mM). After pouring the media into petri dishes, the plates were left to solidify at room temperature. Following solidification 50% of the plates were incubated at 37°C° for 5 days, whilest the other half was incubated at 25°C for10 days.

2.3.1.6.2 Induced Mutations.

Treatment With NTG (N-methyl-N-Nitronitrosoguanidine).

Treatment with NTG was according to Hynes and Pateman (1970 a). Mutagenesis using NTG was performed by suspending conidia in 20 ml of 0.1 M sodium orthophosphate buffer (pH 6.0), and 5 ml saline Tween 80 solution. 2.5 mg of the mutagen (NTG) was very carefully added to the suspension, and the mixture was incubated at 37°C for 35 min. This was followed by centrifugation in a bentch top centrifuge (MSE Mistral 1000) at 3000 rpm for 10 min. The pellet was washed three times with sterile distilled water, and resuspended in water containing the Tween 80 solution. Finally the suspension was treated exactly the same as for spontaneous mutation treatment, using the same nitrogen-sources and selecting at both temperatures (25°C and 37°C) with all nitrogen-sources used.

Treatment With DEO (1,2, 7, 8-Diepoxyoctane).

Isolation Of Chlorate Resistant Mutants.

Mutagenesis with DEO was performed by using the same

procedure for NTG, except where 0.15 ml of the DEO were added to the conidial suspension. After mutagenising the spores, the resuspended pellet of each strain was treated as follows:

a) The treated wild-type suspension was plated on (200 μl/plate), Minimal media containing vitamins solution, sodium chlorate (300 mM), sodium deoxycholate (0.08%) and a sole nitrogen source (10 mM). Two nitrogen sources were used, uric acid and glutamate, and the selection for the mutants was carried out at both temperatures (25° C and 37°C).

b) The treated suspension from the rest of the strains (SAA1040, SAA1032, SAA1023 all *gdhA-niaD* and SAA1023-*gdhB-niaD*) was plated on (200 μl/plate), glucose supplemented Minimal media containing vitamins solution, Sodium Chlorate (300 mM), Sodium deoxycholate (0.08%), Methionine (2 mM) and a sole nitrogen source. Three different nitrogen sources were used with each strain, (except the *gdhB-niaD* strain where only ammonium and proline were used), Proline (10 mM), ammonium (10 mM) and urea (5 mM), and the selection of mutants was at 37°C only. Although, all treatments were carried out under the same conditions, no viability tests were made for the wild-type strain (*yA2, pyroA4*), or SAA1032 and SAA1023 [*gdhA niaD*] transformants. Whilst the viability was checked for the wild-type strain (*biA1*), (SAA1040) [*gdhA-niaD*] and SAA1023 [*gdhB niaD*] transformants. More than 90% of the conidia were killed after the treatment with both mutagens (NTG and DEO). Percentages of survival for each tested strain are shown in Table 2.1.

Table 2.1. Viability Of Mutagenised And Unmutagenised Cells After NTG And DEO Treatments.

Treatment	NTG	DEO			Unmutagenised		
Strain	wild-type[a]	wild-type	SAA1040[b]	SAA1023[c]	wild-type	SAA1040	SAA1023
Survivals/ml	3.4×10^8	5×10^6	8.5×10^7	2.85×10^7	5.96×10^9	9.05×10^8	1.5×10^8
% of survivals	5.7%	0.084%	9.4%	19%			

[a] wild-type *biA1* strain was used in this treatment.

[b] SAA1040 : *yA2, methH2, niaD53, argB+* [*gdhA-niaD*], transformant number 9-6.

[c] SAA1023 : *yA2,wA1, methH2, niaD18, argB+* [*gdhB-niaD*], transformant number 20.

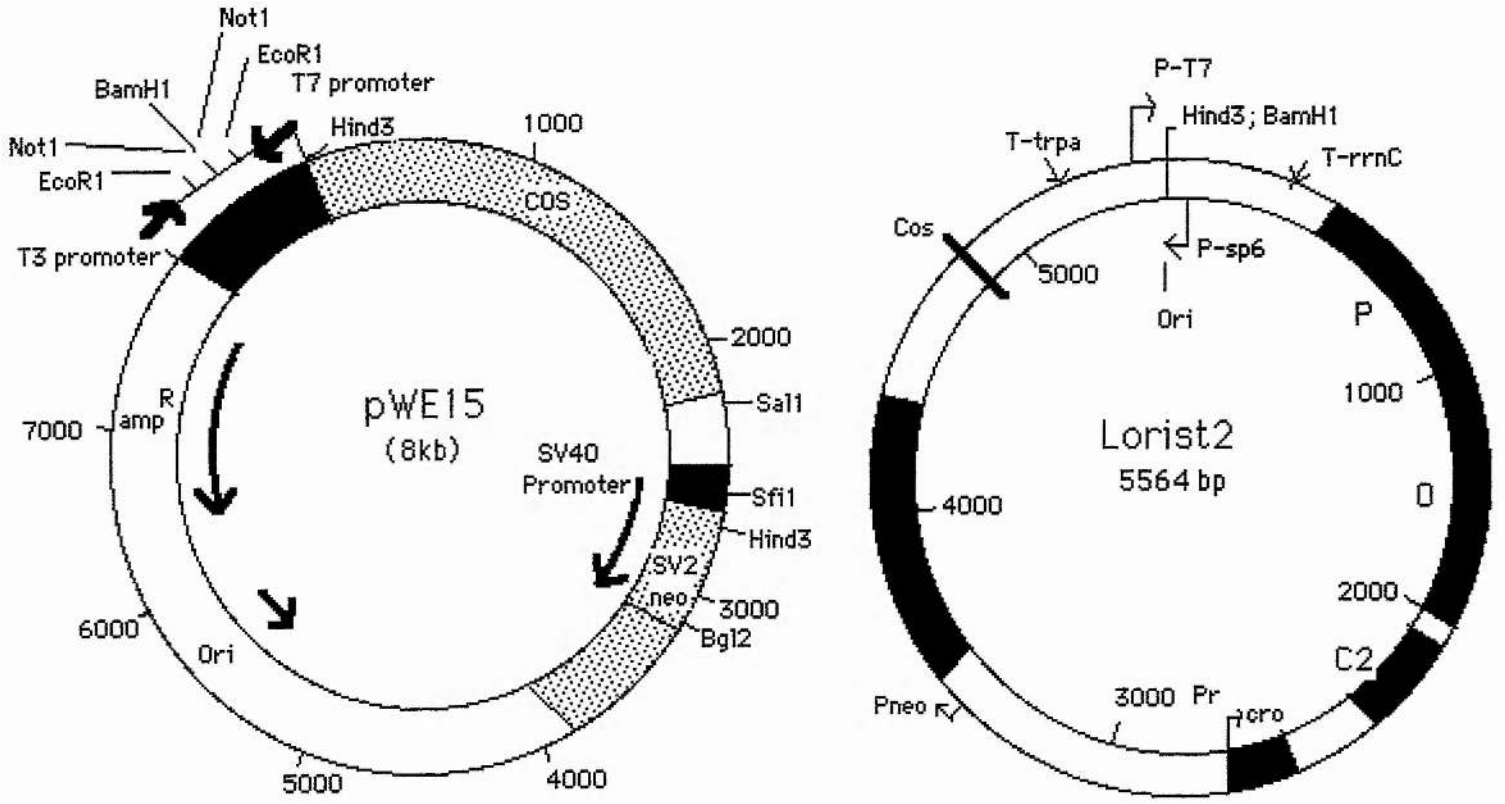


Figure 2.2. Vectors pWE15 And LORIST2, Were Used For The Construction Of The *Aspergillus nidulans* Cosmid Library. pWE15 contains the ampicillin resistance gene, whilst LORIST2 carries the kanamycin resistance gene.

2.3.1.7 Sexual Crosses

Meiotic recombination procedures were used for the genetic analysis of *crn* mutants. Therefore sexual crosses were set up between two different haploid strains with different conidiospore colour markers. Sexual crosses were performed on 2/3 full plates of solid minimal media without a vitamins solution, and sodium nitrate at a concentration of 10 mM (so-called 'crossing plate'). Both strains usually contained a vitamin requirement which helped to force the strains to mate. Under sterile conditions a loopful of liquid complete media was transferred by sterile loop to the centre of the crossing plate. A small conidia from one of the strains (parent 1) was transferred to the crossing plate by sterile loop and suspended in the liquid complete drop. A small conidia from the second strain (parent 2) was transferred in the same way, mixed with parent 1 in a wheel pattern, and spreaded in a star shape through the whole plate. The plates were sealed with masking tape, in order to provide anaerobic conditions which would force a heterokaryon to form between complementing auxotrophs and this enhances the proportion of hybrid fruiting bodies to selfed ones. The plates were incubated at 34°C for 2 weeks. The mature cross was easily viewed under the dissecting microscope (Kyowa, Japan) , and the mature cleistothecia were appeared as black shiny spheres. Several cleistothecia (between 6 to 12) were picked off using a sterile needle from a mixed area of both strains. These were transferred to a further plate of 4% in sterile agar water . Cleistothecia were rolled gently across the 4% agar plate

in order to remove the contaminating hyphae and/or conidiospores [agar at 4% was used to provide a solid support for this rolling procedure]. Individual cleistothecium were taken and crushed on the side of a 5 ml sterilin tube (containing 4 ml of sterile saline / Tween 80 solution) in order to release the haploid ascospores within the cleistothecium. For a rapid test of a cleistothecium which had been truly crossed (selfed or crossed) 10 μl of ascospores aliquot from each cleistothecia suspension was streaked on a quarter of complete plate and incubated at 37°C until conidial colour can be distinguished. 200 μl from each hybrid cleistothecium suspension which had been truly crossed (having both colour markers of parents) were streaked on solid complete media, and incubated at 37°C for 3 days.

Selfed fruiting bodies arrising from a homothalic cross always gave rise to a single colour growth, whilst the hybrid fruiting bodies arising from a heterothalic cross produced a combination of colours depending on the parental strains markers. A hybrid fruiting body from parental strains carrying for example the wild-type (green) marker, and the other a yellow marker would produce two colours ie. yellow and green in a ratio of 1:1. A hybrid fruiting body from parental strains one of them carrying yellow marker, and the other carrying a white marker would give rise to 3 colours of colony, white, yellow, and green (wild-type) in the ratio of 2:1:1, respectively. Progeny from each colour in the cross were picked in the ratio of 1:1 using a sterile needle and inoculated in an upside down position (to avoid scattering and contamination) onto a grided

master plate (complete medium), divided into 26 positions according to a replication unit. The wild-type and both parents of the cross were inoculated also in the same master plate. The plates were incubated at 37°C for 3 days. By using a replication apparatus unit, the progeny and controls were replicated from the master plate into different kinds of growth tests media. Such growth tests were performed to determine the phenotype of recombination and required for the identification and isolation of recombinant strains.

2.3.2 *Escherishia coli.*

2.3.2.1 Growth And Storage Of Strains.

Several *E. coli* strains were used (see section 2.1.2 *E. coli*) for bacterial transformation, preparation of plasmid and cosmid DNA. All cultures were grown at 37°C. For short-term storage *E. coli* was streaked on Luria agar plates either with or without antibiotic (as required). A single colony was used to re-streak fresh solid Luria media at two months intervals, and the plates were kept at 4°C. For long-term storage of strains, a single colony was used to inoculate 5 ml of Luria broth either with or without antibiotic. The cells were incubated at 37°C overnight in a orbital shaker, at speed of 200 rpm. The cells were centrifuged at 4°C, 5000 rpm for 5 min in a bench top centrifuge, and the pellet was resuspended in 5 ml Luria broth. The cells were centrifuged as before and resuspended in 1.5 ml Luria broth. 0.5 ml aliquots were mixed with 2.5 ml of sterile 50% (v/v)

glycerol. The mixture was rapidly frozen in liquid nitrogen for 30 min, and transferred to -70°C. The cells were revived by transferring a small sample from the surface of the frozen material to a Luria agar plate. The frozen samples were immediately returned to -70°C.

2.3.2.2 Plasmids And Cosmids Used In Fungal Transformation.

Plasmids pHElP, DHG25, ARp1, (Figure 1.6) discussed in the introduction) were supplied by Dr. J. A. Clutterbuck (University of Glasgow, Scotland, UK). The chromosome specific *Aspergillus nidulans* cosmid library which was made available to us was constructed as described by Brody *et. al.*, (1991). This library has been used to isolate specific genes successfully (Timberlake *et al.*, 1985). The cosmid clones were constructed in two vectors, the first: is the ampicillin resistant pWE15 (Wahl *et al.*, 1987). The second: pLORIST (Figure 2.2), which carries a kanamycin resistant marker (Gibson *et. al.*, 1987) .

2.3.2.3 *E. coli* Competent Cells.

NB: The method used was based on that described by Sambrook *et. al.*, (1989).

a) One colony of *E.coli* strain DH5α was used to inoculate 5 ml Luria broth (no ampicillin added) and grown overnight in an orbital shaker at 37°C, and 200 rpm shaking speed.

b) 200 ml Luria broth were inoculated with 1 ml of the overnight culture , and the cells were grown as before at 37°C until the O.D was about 0.25 (after approx 3 h).

c) The suspension was centrifuged at 3000 rpm (Sorval RC5C, SS34 rotor) for 5 min, 4°C, the pellet was resuspended very gently, in 100 ml of 100 mM cold $MgCl_2$, 5 mM Tris-HCl pH 7.4 (while keeping cells cold in ice)

d) The suspension was centrifuged at 3000 rpm for 5 min, 4°C.

e) The cells were resuspended gently in 100 ml of 100 mM $CaCl_2$ incubated at 4°C, 5 mM Tris-HCl, pH 7.4.

f) The cells were kept on ice, for 60 min, centrifuged at 3000 rpm, 4°C for 5 min.

g) The cells were resuspended in 1 ml of 100 mM $CaCl_2$, 5 mM Tris- HCl, pH 7.4, 14% glycerol.

h) Dispensed each 100 ml of suspension into pre-cooled (- 70°C), Eppendorf tubes, freezed immediately in liquid nitrogen , stored at - 70°C .

NB: Before use the cells were allowed to thaw in ice. All centifugation steps carried out by using the refrigerated Sorval centrifuge with GS3 rotor for big volumes (above 30 ml) or SS34 rotor for smaller volumes (≤ 30 ml).

Inorder To Check The Transformation frequency Of Competent Cells (should be at least x 10^6 transformants per μg plasmid) The Following Procedure Was Adopted.

a) Two controls were used in each test. First, negative control where

the, bacteria that received no plasmid DNA at all. Second, positive control, where the bacteria that received a known amount of standard preparation super coiled plasmid DNA (e.g circular/supercoiled pUC13, concentration 948 μg/ml)

b) Two Eppendorf tubes each containing 100 ml *E.coli* cells were transferred from -70°C incubator to ice, and left to thaw.

c) 10 ng of plasmid pUC13(i.e 1 ml of 948 μg/ml stock + 94.8 ml TE; 1 ml = 10 ng), were transferred to 100 ml competent cells in ice while the other100 ml aliquot of cells was left without DNA (as the negative control).

d) *E.coli* transformation was carried out with both controls (see *E.coli* transformation procedure, section 2.3.2.4).

e) 10 μl of the positive control suspension were spread on Luria agar containing ampicillin, whilst the negative control was spread twice on agar media with and without antibiotic.

f) After counting the total number of *E.coli* transformants on the positive control plate, and multiplying the number of colonies by the total vol used and the total amount of DNA used, the final reading should be the number of transformants per μg plasmid.

e.g when the total number of colonies was found to be 153, then 153 x 100 (10 μl used from 1 ml total vol) x 100 (10 ng used from 1 μg)= 1.53 x 10^6 transformants per μg plasmid.

2.3.2.4 *E. coli* Transformation.

a) The plasmid DNA (No more than 50 ng in a vol of 10 μl or less) was added to each tube containing 100 μl of *E. coli* competent cells.

b) The contents were mixed by swirling gently, and the tubes left on ice for 30 min.

c) The tubes were placed in a receprical water bath that has been preheated to 42°C for 90 sec, without shaking , then transfered to ice for 2 min.

d) 900 μl Luria broth were added to each tube, and incubated for 45 min at 37°C in a orbital shaker (at 125 rpm or less) to allow the bacteria to recover (and to express the antibiotic resistance marker encoded by the plasmid).

e) 200 μl of transformed competent cells were transfered onto Luria agar containing the appropriate antibiotic for selection, using a sterile blend glass rod, gently the transformed cells were spreaded over the surface of the agar plates.

f) The plates were left at room temperature until any liquid was dried up, and the plates were incubated in inverted position at 37°C for 18 h.

* Control 1: bacteria that received a known amount of the standard preparation of super coiled plasmid DNA.

** Control 2: bacteria that received no plasmid DNA at all.

2.3.2.5 Rapid Preparation Of Plasmid.

NB: In order to check if the bacterial colony contains any inserted DNA in the plasmid, the following approach was used.

a) Using a sterile toothpick, the bacterial colony was lysed in 20 μl sterile distilled water, and the remaining on the toothpick was cultured on Luria broth containing ampicillin plate as stock.

b) Another 20 μl of cracking buffer were added to the lysed cell and mixed gently.

c) The entire vol (ie. 40 μl) was loaded onto 0.8% agarose gel, and ran at 50 volts (carefully ensuring that the level of 1 x TAE buffer does not cover the gel's wells).

d) After the dye has moved away from the well, more 1 x TAE buffer was added to cover the gel surface.

e) After several hours the gel was examined for DNA under a U.V Transilluminator.

2.3.2.6 Plasmid Mini Preparation Using Qiagen Spin Column-50.

a) *E.coli* transformants parbouring a plasmid, were collected by sterile toothpick and suspended by swirling in 250 μl P1 (resuspension buffer of 50 mM Tric-HCl, and 10 mM EDTA, pH 8.0) to dissolve completely.

b) 250 μl of P2 (Lysis buffer) was added to the suspension mixture and mixed by gentle inversion 6 times, and incubated at room temperature for 5 min.

c) 350 μl of chilled buffer N3 (Neutralising buffer) were added to the suspension, followed by immediate gentle inversion to prevent precipitation, then incubated on ice for 5 min.

d) After the previous step, the suspension was centrifuged for 10 min, at 14000 rpm in 1.5 ml Eppendorf tube.

e) A Qaigen column-50 was placed in the special Qaigen collecting 2 ml Eppendorf, the suspension was then transferred by pipette to this column.

f) The supernatant was centrifuged for 60 sec at 14000 rpm.

g) After centrifugation, the supernatant was drained flow through fraction from the 2 ml collecting Eppendorf, and the column was placed in it again.

h) The column was washed with 0.5 ml of buffer PB and centrifuged for 60 sec, followed by drain flow through fraction.

i) Following this treatment the column was washed by 0.75 ml of buffer PE (Wash buffer) and centrifuged for 60 sec.

j) The PE buffer was drained off , centrifuged again for an additional 60 sec to remove residual wash buffer.

k) The column was placed in 1.5 ml Eppendorf tube, the DNA eluted by adding 100 μl of 100 mM Tris-HCl, pH 8.5 or TE pH 8.5, and centrifuged for 60 sec.

l) After centrifugation,the DNA concentration was checked by running on an 0.8% agarose gel with, 1 X TAE buffer at 25 volts for few hours.

2.3.2.7 Plasmid Mini Preparation.

a) a single colony of an *E. coli* transformants was grown in 2.5 ml of Luria broth containing ampicillin (100 μg/ml) at 37°C overnight, at 200 rpm in a orbital shaker.

b) The 2-5 ml culture was centrifuged at 6000 rpm for 5 min in Eppendorf micro centrifuge.

c) The cells were resuspended in 100 ml of solution I by inverting the tube several times.

d) 200 μl of solution II were added, and mixed by inverting the tube, the mixture was incubated in ice for 10 min.

e) 150 μl of 3 M KOAC, pH 4.8, were added, mixed gently, placed in ice for 10 min.

f) After centrifugation,the suspension was centrifuged for 10 min at 14000 rpm.

g) The supernatant was then transferred to sterile tube, and the same vol of phenol : chloroform : isoamylalcohol (25:24:1) was added, mixed, and centrifuged for 10 min at 14000 rpm.

h) The same vol of Chloroform: Isoamylalchohol (24:1), was added ,mixed, and centrifuged for 10 min at 14000 rpm.

i) After this treatment, 1/10 vol of 3 M KOAC and 2 vol of 100% cold (4°C) ethanol was added to the supernatant, and incubated at -20°C for 20 min.

j) After this incubation step, the suspension was centrifuged for 10 min at 14000 rpm.

k) The DNA was washed with 70% cold ethanol, dried and dissolved in 20 μl TE buffer.

l) RNase were added to a final concentration of 10 μg/ml, and incubated at 37°C for 1 h.

m) After incubation, the concentration of DNA was checked by running on an 0.8% agarose gel with 1 X TAE buffer, at 25 volts for a few hours, by using 2 μl DNA in a total vol of 20 μl.

2.3.2.8 Plasmid Midi Preparation Using Qiagen 100-Tip Column.

a) A single colony of *E. coli* transformants was grown in 5 ml Luria broth containing ampicillin (100 μg/ml) overnight, at 37°C, in a orbital shaker at 200 rpm.

b) One ml of this overnight culture, was inoculated to either 50 ml or 100 ml of Luria broth containing ampicillin (depending on the copy number of the plasmid). This culture was grown at 37°C overnight at 200 rpm in a orbital shaker.

c) The cells were centrifuged for 10 min, at 4°C, at 5000 rpm (Sorval RC5C, SS-34 rotor) .

d) The pellet was resuspended in 4 ml of solution I (resuspension buffer).

e) 4 ml of solution II (lysis buffer) were added, and mixed gently, incubated at room temperature for 5 min.

f) 4 ml of buffer 3 (KOAC, 3 M) were added to the mixture, and mixed gently, incubated in ice for 15 min.

g) The cells were centrifuged at 4°C for 30 min at 12000 rpm, the plasmid eluate (supernatant) was kept in another tube in ice.

h) A Qiagen 100-tip column was equilibrated with 4 ml QBT buffer(equilibration buffer).

i) The plasmid eluate was applied to the column, the column was washed twice

with 10 ml QC (wash buffer) buffer.

j) The plasmid was eluated with 5 ml QF (elution buffer) buffer .

k) The DNA was precipitated with 2.5 volume of 100% ethanol, and incubated at 4°C.

l) This DNA suspension was centrifuged at 12000 rpm at 4°C for 30 min.

m) The resulting pellet was washed with 5 ml of cold 70% ethanol, and left to dry for 5 min at room temperature.

n) DNA was resuspended in 50 μl of TE buffer. The concentration was determined on 0.8% agarose gel with, 1 X TAE buffer, ran at 75 volts for 3 h.

2.3.2.9 Large-Scale Plasmid Preparation.

Solutions And Buffers Required.

Solution I (Resuspension Buffer).

50 mM glucose, 25 mM Tris- HCl pH8.0, 10 mM EDTA.

Solution II (Lysis Buffer, prepared fresh).

1 ml from 2 M NaOH, 1 ml from 10% SDS and continue to 10 ml with sterile H_2O.

TE Solution (Tris-EDTA Suspension Buffer).

10 mM Tris- HCl PH 8.0, 1 mM EDTA pH 8.0.

QBT Buffer (Equilibration Buffer).

750 mM NaCl, 50 mM MOPS pH 7.0, 15% ethanol, 0.15% Triton X-100

QC Buffer (Wash Buffer).

1 M NaCl, 50 mM MOPS PH 7.0, 15% ethanol.

QF Buffer (Elution Buffer).

1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% ethanol.

Saline Tween 80.

0.01% Tween 80, 0.9% NaCl.

Plasmid Preparation.

a) A single colony of an *E. coli* transformant was grown in 5 ml Luria broth containing ampicillin (100 μg/ml) overnight at 37°C at 200 rpm.

b) One ml of this overnight culture was inoculated into 200 ml Luria broth containing ampicillin, and grown at 37°C overnight at 200 rpm.

c) The cells were centrifuged at 4°C for 10 min, at 5000 rpm.

d) After centrifugation, the pellet was resuspended in 4 ml solution I (ice cold).

e) The suspension was transferred to 30 ml sterile plastic centrifuge tubes, and covered with sterile lids.

f) The suspension was incubated at room temperature for 5 min.

g) After incubation, 8 ml of solution II were added and the solution kept in ice for 5 min.

h) Then 6 ml of 3 M KOAC, pH 5.0 were added, mixed, and incubated in ice for 20 min.

i) The cells were centrifuged for 40 min at 12000 rpm in a orbital shaker, at 4°C.

j) After centrifugation, 0.7 vol of isopropanol was added to supernatant, mixed, and incubated at room temperature for 20 min.

k) After incubation, the suspension was centrifuged for 20 min, at 12000 rpm, at 20°C.

l) The pellet was washed with 70% ethanol, dried and dissolved in 4.5 ml TE solution.

m) 2 μl of (final concentration 10 mg/ml) RNase were added and incubated at 37°C for 60 min.

n) After incubation 0.5 ml of 1 M Mops (3-N-Morpholinopropane sulfonic acid),

pH 7.0 and 1 ml of 5 M NaCl were added, and the mixture kept at room temperature.

o) A Qiagen-tip 500 column was equilibrated with 10 ml QBT buffer, then the plasmid solution was transferred to the column, and left to drain through.

p) The column was washed with 10 ml QC buffer twice.

q) The plasmid was eluated with 15 ml QF buffer, and the eluate was collected in a further sterile tube.

r) To the plasmid eluate, 0.7 vol of isopropanol was added, mixed, and incubated in ice for 20 min (2 vol of 100% ethanol previously stored at -20°C could be used).

s) Plasmid DNA was centrifuged for 20 min at 12000 rpm, at 4°C.

t) The pellet was washed with 70% ethanol, dried, and resuspended in 100 μl TE solution.

2.3.2.10 Plasmid Preparation Using Caesium Chloride Method.

a) A single colony of an *E. coli* transformants was grown in 5 ml Luria broth containing ampicillin (100 μg/ml) overnight at 37°C in a orbital shaker at 200 rpm.

b) One ml of this overnight culture was used to inoculate 200 ml Luria broth containing ampicillin and grown at 37°C overnight at 200 rpm.

c) The resulting cells were centrifuged at 4°C for 10 min at 5000 rpm (Sorval RC5C, SS-34 rotor).

d) After centrifugation the pellet was resuspended in 4 ml ice- cold solution I (resuspension buffer).

e) The suspension was transferred to 30 ml sterile plastic centrifuge tubes and covered with sterile lids.

f) Such tubes were incubated at room temperature for 5 min.

g) After incubation 8 ml of solution II (lysis buffer) were added and kept in ice for 5 min.

h) Then 6 ml of 3 M KOAC buffer, pH 5.0 was added, mixed, and incubated in ice for 20 min.

i) After incubation the suspension was centrifuged for 40 min at 12000 rpm, and 4°C.

j) After centrifugation 0.7 vol of isopropanol was added to the supernatant, mixed, and incubated at room temperature for 20 min.

k) The cells were centrifuged for 20 min at 12000 rpm at 20°C.

l) The pellet was washed with 70% ethanol, and left to dry.

m) 6 ml of TE solution were added to the pellet, and placed in a 30°C water bath to dissolve.

n) For each 1 ml of TE solution, 1 gm of CsCl was added. This mixture was placed in a 30°C water bath to dissolve (when the total volume did not reach 12 ml, another 6 ml TE and 6 g CsCl were mixed in a separate tube to increase the vol).

o) 0.96 ml of EtBr (10 mg/ml) solution was added to the 12 ml TE and CsCl, and centrifuged at 8000 rpm for 5 min, at room temperature using SS-34 rotor.

p) The clear red solution under the scum float (complex of EtBr and bacterial proteins) were transferred by a syringe to a further sterile tube.

q) A Beckman Quick-seal (CsCl density gradient tubes), were weighed with caps on a sensitive electronic balance and pairs of tubes with approximately the same weight centrifuged as follows.

r) The tubes were placed in the centrifuge holder, and a sterile blue tip was fitted to the tube .

s) Using a glass pipette the solution was transferred very gently to a further tube , without developing air bubbles, until the tube was full (if not full in the first instance,light paraffin oil was used to completely fill the tube).

t) Each tube was weighed alone, and any excess vol was absorbed with tissue paper.

u) The tubes were sealed, and centrifuged for 60 h, at 20°C and 53000 rpm (Sorval ultracentrifuge, OTD65B).

v) After centrifugation the tubes were placed separately on a special stand, and a U.V transilluminator used to reveal a floating band of circular plasmid approximately near the middle of the tube. An upper band was apparent (linear DNA), and a top band (bacterial protein).

w) Using a heated syringe needle, a hole was made near the neck of the tube in order to release the pressure inside. Another needle was

inserted into the tube beneath the circular plasmid band, and the band was withdrawn and transferred to a further tube.

x) In order to wash the DNA from the excess of EtBr, 2 ml of TE solution and 2 ml of butanol were added, mixed gently, and incubated at room temperature for 2 min. Two bands were formed, an upper band of butanol and EtBr, and a lower band of plasmid in TE solution.

y) Plasmid was transferred to a further tube, and the washing step was repeated

6 times until the plasmid suspension appeared colourless under the U.V light.(EtBr free)

z1) In order to clean the DNA from caesium salts, the DNA was dialysed by placing the dialysis bags filled with the DNA suspension in a beaker containing 2 litres of TE solution. The beaker with the contents was incubated in a cold room with low stirring for 2 h.

z2) The dialysis bags were transferred to a further 2 litres TE solution for another 2 h, and finally transferred to a further 2 litres TE solution and left overnigh.

z3) 1/10 vol of 5 M NaCl, and 2 vol of cold 96% ethanol, were added to the DNA solution. The tubes were incubated at -20°C overnight in order to precipitate the DNA.

z4) The DNA was centrifuged at 4°C, at 12000 rpm, for 30 min. The resulting pellet was washed with 70% cold ethanol, dried, and resuspended in 100 μl TE solution.

z5) DNA concentration was determined by running on a 0.8% agarose, with 1 x TAE buffer, at 75 volts for 3 h.

2.3.2.11 Phenol, Chloroform, Isoamyl alcohol And Ethanol Precipitation Of DNA.

a) An equal vol of phenol : chloroform : isoamylalchohol (24:25:1) was added to the diluted DNA solution, mixed gently, and centrifuged for 10 min at 3000 rpm.

b) An equal volume of chloroform:Isoamylalcgohol (24:1), was added to the supernatant, mixed, and centrifuged for 10 min at 3000 rpm.

c) After centrifugation the supernatant was transferred to a further tube, where 1/10 vol of 3 M KOAC buffer, and 2 vol of 100% cold ethanol (stored at -20°C) were added, mixed gently, and incubated at - 80°C for 15 min.

d) The solution was centrifuged for 15 min at 14000 rpm.

e) After centrifugation the DNA was washed with 70% cold ethanol, dried, and resuspended in sufficient vol of TE buffer.

2.3.2.12 Southern Blot.

Denaturing Solution.

1.5 M NaCl

0.5 N NaOH

Neutralising Solution.

0.5 M Tris-HCl, pH 7.4

1.5 M NaCl

20 X SSC:

NaCl	175.3 g
Na citrate	88.2 g

The pH was adjusted to 7.0 with 5 M NaOH, and the total vol was made up to 1 litre with distilled water. After autoclaving the solution was stored at room temperature.

20 X SSPE	NaCl	175.3 gm
	NaH_2PO_4	27.6 gm
	EDTA	7.4 gm

The pH was adjusted to 7.4 with 5 M NaOH, and the total vol was made up to 1 litre with distilled water. After autoclaving the solution was stored at room temperature.

Southern Blot Procedure.

a) DNA of interest was ran on an 0.8% agarose gel, with 1 X TAE, at 75 volts for 4 h.

b) The gel was photographed with a ruler alongside, the top right hand corner of the gel was nicked in order to orientate.

c) The gel was incubated in 0.25 M HCl, and shaked gently (100 rpm) at room temperature for 30 min.

d) after pouring off the HCl, the gel was washed with distilled water, and the denaturing solution was added and placed on a reciprocal shaker for 40 min.

e) After discarding the denaturing solution, the gel was washed with distilled water, then neutralising solution was added, and placed on a reciprocal shaker for 50 min.

f) The neutralising solution was discarded and the gel was washed with distilled water.

g) For blotting, the following were prepared.

- 2 pieces of 3 MM chromatography paper 3 times the length of the gel, and about 2 cm wider for the wick.
- 1 piece of Hybond N membrane, the same size as the gel.
- 6 pieces of 3 MM paper, the same size as the gel.
- wad of absorbent towels roughly the same size as the gel.
- 20 X SSC solution, prespex bridge, and glass dish.

h) Two layers of 3 MM paper (=wick), were soaked in 20 X SSC solution, and placed on glass bridge (any trapped air bubbles were removed by glass rod).

i) The gel was washed first in distilled water, placed inverted on wick, and in order to prevent the absorbent towels from touching the wick the edges of the gel were covered with clingfilm or nescofilm.

j) The piece of Hybond N membrane (size of the gel) was placed on the top of the gel, and the corner which matches that of the gel was nicked.

k) The 3 pieces of 3 MM paper were soaked in 20 X SSC solution, and placed on the top of the membrane (any air bubbles were rolled out by glass rod).

l) The remaining pieces of dry 3 MM paper were placed on the top, and a wad of absorbent towels was applied.

m) A piece of perspex was placed on the top, and blot overnight.

n) On the following day pieces of the towels and the 3 MM paper were removed, and the membrane was washed briefly in 2 X SSC

solution to remove any particles of gel, and left to dry at room temperature.

o) The DNA was cross linked for 4 min using the U.V transilluminator.

p) To determine that DNA transfer has been completed, the gel was restained with EtBr, and exposed to U.V light.

q) The filter was rapped in clingfilm and incubated at -20°C, for hybridisation.

2.3.2.13 Preparation Of Chromosome III Cosmid Bank Filters.

a) A piece of Hybond N or magna Nylon- membrane was divided into 10 x 20 squares, and placed on top of both Luria agar containing ampicillin and Luria agar containing kanamycin.

b) Individual cosmid clones of chromosome III were inoculated on both Luria agar containing ampicillin, and Luria agar containing kanamycin using sterile tips on top of membrane (antibiotic used depends on the resistant marker carried on the plasmid).

c) The plates were incubated in bacterial incubator at 37°C for 20 h.

d) After incubation five large plastic trays were prepared, and five pieces of 3 MM chromatography paper were cut according to the tray's base, where one paper was placed in each tray.

e) Sufficient vol of 10% SDS was added to the first tray, just to wet the 3 MM paper, the excess amount was discarded.

f) The Hybond N membrane was placed on top of 3 MM paper using forceps, where the bacterial colonies were facing the upperside, and left for 3 min at room temperature.

g) The membrane was transferred to the second tray, which has been wetted with sufficient vol of denaturing solution, and left for 10 min.

h) After denaturing cells, the membrane was transferred to the third tray, which was wetted with sufficient vol of 1 M Tris-HCl, pH 8.0 and left for 5 min.

i) The previous step was repeated in order to make sure that the cells were neutralised.

j) The membrane was transferred to a 5th tray, where the 3 MM paper was wetted with sufficient vol of neutralising solution and left for 5 min.

k) After neutralisation of cells the membrane was transferred to a clean dry 3 MM paper, and left to dry completely at room temperature.

l) The membrane was transferred to a cross-linker for 4 min (with the U.V Transilluminater colonies were facing the U.V light).

2.3.2.14 DNA Hybridisation.

NB: All solutions and tips were sterilised by double autoclaving at a pressure of 15 pounds per square inch, at 121°C for 15 min. All glass wares were sterilised in an oven at 200°C for 8 h. Labelling procedure was according to that described by Sambrook *et. al.*, (1989).

RNA Polymerase Storage Buffer.

20 mM K_2PO_4, pH 7.7

0.1 M NaCl

0.1 mM EDTA
1 mM DTT
50% (V/V) Glycerol
0.01% (W/V) Triton X-100.

5 X T_3/T_7 RNA Polymerase Buffer.

0.2 M Tris-HCl, pH 8.0
40 mM $MgCl_2$
10 mM Spermidine-$(HCl)_3$
125 mM NaCl.

Probe Labelling.

a) DNA probe used (e.g. *meaB* cosmid W02E01/*Hae* III; 50 ng/20 μl SDW) was denatured by boiling for 2 min, then incubated immediately in ice to prevent reannealing of strands.

b) Labelling was proceeded as in Amersham Megaprime kit using 30 mCi $\propto$ ^{32}P dCTP.

Labelling Mixture At Room Temperature.	Vol (μl)
5 X buffer	8
100 mM DTT	4
RNase	1.2
0.5 mM ATP GTP, UTP	8

100 μM CTP	4.8
DNA	2

d) The total vol (28 μl) was divided into two halves, where 4 μl of $\propto$ ^{32}P CTP (100 μM) and 2 μl (1u/μl) of T3 polymerase were added to the first half, and 4 μl of $\propto$-^{32} P-CTP, 1 μl T7 (1u/μl) polymerase and 1 μl DEP H_2O were added to the second half (total of 20 μl each).

e) The tubes were incubated at 37°C for 60 min , 0.25 μl of RNase-free DNase (7.5 u/μl) were added and the mixture was incubated at 37°C for 15 min .

f) The labelled probe was separated from the unincorporated nucleotides by filtration through Sephadex G 50 using NICK columns (Pharmacia).

g) After pouring the preservative solution of the Nick column, it was placed on a stand behind the shield, and filled with DEP TE (10:1) solution and left to drain off (Washing).

h) 20 μl of T3 or T7 RNA polymerase labelled probe and 30 μl of DEP TE solution were applied to the NICK column in addition 5 μl of DEP blue dextran was added to the top (column capacity 50 μl) .

i) The blue fraction containing the labelled DNA was collected, where a total of 8 fractions were collected 3 drops in each, except the last tube where approx 1.2 ml were collected.

j) A mini-monitor (Geiger counter) was used to estimate the number of counts in each fraction.

Fraction Number	Geiger counter Counts
1	0

2	20
3	120
4	80
5	40
6	75
7	130
8	> 1000

k) There was two peaks of counts. The first in the blue fraction which contains the probe, the second is the unincorporated nucleotides.

l) The percentage of incorporation was estimated by dividing the total number of counts in the blue fraction by the total number of counts obtained, multiplying by a 100.

Pre-Hybridisation

Prehybridisation Solution:

Stock	Volume (ml)	Final Conc
20 X SSPE	12.5	5 X
30% PEG 6000	10.0	6.0%
10% Skimmed milk	2.5	0.5%
10% SDS	5.0	1.0%
5% $Na_4P_2O_7$	1.0	0.1%
5 mg/ml herring sperm DNA	2.5	250 μg/ml

SDW 16.5

a) The already cross-linked membranes (southern blot) were wetted with DEP water in sterile dishes by gentle shaking.

b) 50 ml of sterile DEP pre-hybridisation solution was added to the membranes, and the membranes were placed in a receprical shaking water bath, at 65°C, at 70 rpm shaking speed for 5 h.

Hybridisation.

a) The labelled probe was added to the membranes in the dishes, shaked gently, and incubated overnight at 65°C.

b) After careful pouring of the hybridisation solution in the radioactive waste disposal sink, membranes were washed briefly with small vol (250 ml) of 5 X SSC, 0.1% SDS, 0.1% $Na_4P_2O_7$. Then approx 200 ml of the same solution were added to the membranes and incubated in a water bath with gentle shaking at 65°C for 60 min.

c) After incubation the membranes were washed again with 2 X SSC, 0.1% SDS, 0.1% $Na_4P_2O_7$.

d) Finally filters were sealed in plastic bags and exposed to autoradiographic film (Fuji RX) in an autoradiography cassette with intensifying screens. The

cassette was stored at -70°C. Exposure was usually overnight, or longer if a stronger signal was required. Upon defrosting of the film cassette, the film was removed and processed in a Fuji RGII X-ray film developer.

NB: If a membrane was to be re-probed, it was first stripped by boiling in 0.1% SDS.

2.3.2.15 Electrophoretic Separation And Northern Blotting Of RNA.

Radiolabelled probing of filters was carried out as in southern blotting. Hybridisation was carried out at 42°C in 50% deionised formamide, 30% 20 X SSPE, 5% 100 X Dehardt's solution, 5% herring sperm DNA (5 mg/ml) and 1% SDS.

Denhardt's Solution.

Gelatin	2%
PVP	2%
Ficoll 400	2%
SDS	1%

High stringency washing was made to 0.2 X SSC, 0.1% SDS, 0.1% sodium pyrophosphate.

TE SDS Solution.

Components	Vol/ml
1 M Tric-HCl (pH 7.5)	1
0.5 M EDTA (pH 8.0)	0.2
10% SDS	1
DEP-SDW	97.8

Agarose Gel For RNA Analysis.

Agarose	0.3 g
100 X MOPS	0.3 μl

10 mg/ml EtBr	0.6 μl
SDW	28.1 ml

After melting and cooling to 50°C, 1.62 ml formaldehyde was added. Before loading 5 μl RNA solution was heated to 95°C for 5 min and the vol was continued to 20μl with RNA loading buffer.

RNA Loading Buffer.

Components	vol(ml)
Deionised formamide	7.2
10 X MOPS	1.6
Formaldehyde	2.6
DEP-SDW	1.8
80% Glycerol	1
Saturated bromophenol blue	0.8

Isolation Of RNA.

a) 4 g fungal mycelia were ground to a fine powder in liquid nitrogen and added to 12 ml GUS:CN and 1.2 ml mercaptoethanol.

b) The suspension was stirred vigorously, homogenised then added to 60 ml of 4 M LiCl.

c) The mixture was incubated at room temperature for 20 min then incubated at 4°C overnight.

d) After incubation the suspension was centrifuged at 2500 rpm for 5 min at 4°C (Sorval, HB-4).

e) The suspension was centrifuged again at 8000 rpm for 90 min at 4°C.

f) The formed pellet was resuspended in 40 ml of 3 M LiCl and centrifuged at 11000 rpm for 60 min at 4°C.

g) After centrifugation the pellet was resuspended in 2 ml TE:SDS solution and extracted with an equal vol of CH_3Cl_3:IAA.

h) After this extraction the phenol phase was extracted with 1 ml TE:SDS solution.

i) The aqueous phase was extracted with CH_3Cl_3:IAA until no interface was visible, and extracted once more with CH_3Cl_3:IAA.

j) After final extraction 0.1 X vol of 3 M sodium acetate, pH 5.2 was added along with 2 X vol 96% ethanol previously stored at -20°C.

k) The solution was incubated at -20°C overnight in order to precipitate the RNA.

l) After incubation the solution was centrifuged at 12000 rpm for 15 min at 4°C.

m) The formed pellet was washed with 90% ethanol and allowed to dry and RNA was resuspended in suitable vol of DEP-SDW.

mRNA Isolation.

mRNA isolation was carried out by means of an mRNA purification kit (pharmacia.cat no.279258-01) and used as per manufacturer's instructions.

RNA Solutions.

All solutions required for RNA preparation were prepared with DEP-treated water and double autoclaved. All glassware were siliconised prior to use.

GUS:CN Solution.

Guanidine Isothiocyanate	5 M
EDTA	10 mM
Tric-HCl, pH 7.5	50 mM

The mixture was dissolved at 50°C, filttered and stored at -20°C and 0.1 X vol B-mercaptoethanol was added prior to use.

Measurement Of Nucleic Acid Concentration.

RNA concentration and purity were determined spectrophotometrically. One O.D at 260 nm is corresponding to approximately 50 mg/ml RNA. i.e. the obtained absorbance at 260 nm X dilution factor X 50 mg/ml will give the RNA concentration in mg/ml. However, the ratio of O.D260/O.D280 gives the purity, where a value between 1.8 to 2 is an indicative of pure RNA. In each experiment all measurements were scored in triplicates and averaged.

2.3.3 *Arabidopsis thaliana*

2.3.3.1 Growth And Storage Of *Arabidopsis thaliana.*

Arabidopsis. thaliana seeds were stored in cellophane paper at 4°C, and grown as mentioned in *Arabidopsis* growth media (see section 2.2.3). Sterile *Arabidopsis* leaves stored at -70°C were transferred from -70°C to liquid nitrogen, and used for DNA preparation as mentioned below.

2.3.3.2 Large-Scale Genomic Plant DNA Preparation.

CTAB Buffer.

140 mM sorbitol, 220 mM Tris-HCl pH 8.0, 22 mM EDTA, 800 mM NaCl, 1% sarkosyl, 0.8% CTAB

The CTAB method, adapted by Dean *et. al.*, (1992) for *Arabidopsis.. thaliana* DNA, has been used as follows.

a) 2 g of sterile leaves were grounded in liquid nitrogen .

b 25 ml of CTAB buffer were added and incubated for 20 min at 65°C with occasional vigorous shaking .

c) After incubation 10 ml of chloroform were added, and the tube was shaken by inverting for 20 min.

d) The mixture was centrifuged at 2800 x g (4700 rpm : SS-34 rotor) for 15 min.

e) After centrifugation 17 ml of isopropanol were added to the supernatant and incubated in ice for 10 min in order to precipitate the DNA.

f) The tube was centrifuged at 2800 x g for 5 min.

g) The precipitate was resuspended in 4 ml TE solution.

h) An equal vol of 4 M LiOA was added, and incubated in ice for 20 min to precipitate the RNA.

i) After incubation the tube was centrifuged at 2800 x g for 10 min.

j) 2 vol of ice-cold ethanol were added to supernatant in order to precipitate the DNA, and incubated in ice for 20 min.

k) The tube was centrifuged at 2800 x g for 5 min.

l) The pellet was dissolved in 0.9 ml TE solution.

m) 100 μl of 3 M NaOA were added and the suspention was purified by :

- phenol extraction,
- phenol: chloroform: isoamylalchohol extraction,
- chloroform extraction.

n) After purification 1/10 vol of 3 M KOAC buffer, and 2 vol of ice- cold ethanol were added with inverting gently, and incubated at -70°C for 15 min. The suspension was centrifuged for 15 min, at 14000 rpm.

o) The pellet was resuspended in TE solution, 10 mg/ml RNase was added and incubated for 1 h at 37°C.

p) DNA concentration was determined by running on an 0.8% agarose gel with 1 X TAE buffer, at 25 volts for 3 h.

2.4 General Molecular Techniques.

2.4.1 Agarose Gel Electrophoresis.

The size and the concentration of the horizontal slab gels was chosen with regard to the nature of the sample needed to be analysed. Usually 0.8% or 2% agarose gels were used. In general mini-gels (40 ml) were used for rapid electrophoresis, and for examining the PCR products (on 2% gel) unless the band of interest needed to be cut from the gel in order to be cleaned and used for sequencing, it was ran again on 0.8% gel. Larger gels (200 ml) were used for a more accurate resolution. The agarose was dissolved in 1 X TAE buffer, melted and allowed to cool down to 50°C.

Ethidium bromide was added to the media at a final concentration of 0.5 μg/ml. The gel was poured into the appropriate gel holder, and the required comb providing the sample wells was placed in its position. The gel was allowed to solidify at room temperature. After solidification of the gel, the comb was removed and the gel was submerged in 1 X TAE buffer. The DNA samples were loaded to the gel wells by using the micropipette, and the electrophoresis was performed at 25 volts (mini gel) for 2 h or 75 volts (midi gel) for 3 to 4 h, depending on the purpose of running the gel (high resolution was achieved at lower voltages whilst fast separation was achieved at higher voltages.

Electrophoresis Buffer.

10 X TAE (Tris-acetate-EDTA). 48.4 gm of Tris-base, 11.42 ml glacial acetic acid, and 20 ml of 0.5 M EDTA. The total vol was continued to 1 litre, and the pH was adjusted to 8.0.

Loading Buffer.

0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water, and the buffer was stored at 4°C.

Cracking Buffer.

2 ml of 5 M NaOH, 2.5 ml 10% SDS, 10 gm sucrose, Bromocrysol green as a dye, and the total vol was made up with distilled water to 50 ml.

Lysis Buffer.

50 mM glucose, 25 mM Tric-HCl pH 8.0, and 10 mM EDTA.

2.4.2 Polymerase Chain Reaction (PCR) Principle.

PCR was used to amplify DNA fragment that lies between two regions of known sequence. In every PCR reaction two oligonucleotides were used as primers for a series of synthetic reactions, that are catalysed by a DNA polymerase. These primers have different sequences (see section 2.4.2.1), and were complementry to sequences that lie on opposite strands of the template DNA and flank the fragment of DNA that is to be amplified. The template DNA was first denatured by heating in the presence of a large molar excess of each of the two oligonucleotides and the 4 dNTPs. The reaction mixture was cooled to a temperature which allows the primers to anneal to their target sequences. After that the annealed primers were elongated with DNA polymerase. The cycle of denaturation, annealing, and DNA synthesis was repeated several times (see section PCR conditions). Because the product of one cycle of amplification serves as templates for the next, each successive cycle doubles the amount of DNA product. The presence of bacterial sequences in putative fungal transformants was confirmed by the ability to amplify such sequences using the PCR method. Different primers (Ampicillin, or lambda (λ)) were used depending on the clone used to transform the fungal strain. The enzyme used was Taq polymerase. For DNA sequencing PCR was used to amplify

fragments of the *cnxH* gene from wild-type genomic DNA. Additionally, DNA fragments of temperature-conditional or non-conditional *cnxH* mutants were amplified for the same purpose. In every single PCR reaction, wild-type (as a positive control) and double autoclaved distilled water (as a negative control) were included. The products of PCR reactions were providing template for cloning, and direct sequencing of PCR amplified regions of the gene. The universal PCR primers used for sequencing wild-type *cnxH* gene were provided by Amersham. Other primers were designed from the sequence of the wild-type gene, and provided by either Amersham or St. Andrews university.

The PCR Method.

PCR reactions were carried out in a total vol of 100 μl, in double autoclaved PCR Eppendorf tube. In order to ensure that a maximal yield of product with the correct size of band is achieved, a series of experiments were carried out to determine the optimum quantity of DNA template (ranging from 1 pg to 1 μg) and the volume of concentrated primer (0.5-1 μl). The reaction conditions for each set of primers were also optimised individually with respect to annealing temperatures, extension times, and cycle number. The PCR reactions were carried out in a HYBAID thermal cycler.

2.4.2.1 Preparation Of Primers.

a) DNA pellet was resuspended in 10 μl sterile distilled water and incubated for 15 min. at room temperature.

b) Primer concentration was estimated spectrophotometrically at 260 nm, and was calculated according to the following equation:

$$\frac{OD_{260} \times \text{Dilution Factor}}{0.01 \times N}$$

N: Primer Base Number.

c) The primer was diluted suitably to a working concentration of 1 pmol/μl, and used for PCR amplification.

Primers Used For Amplification Of *A nidulans* Transformants.

Primer Used	Primer sequence	Annealing Temperature
AMP1	CTG TGA CTG GTG AGT AC	52°
AMP2	CAA CAT TTA CGT GTC GC	52°
λ1	GTT CTC AAT TTC AGC ATC C	54°
λ2	GAC AGG TGC TGA AAG CG	54°
AMP1- AMP2	2165 - 2477	312 bp (Acc.no.x02514)
λ1 - λ2	120 - 367	247 bp (Acc.no. voo636)

AMP1+ AMP2 carried on pUC, pBluescript, pWE15 vectors.

λ1 + λ2 carried on pWE15 vector.

The primer is up to 20 - 22 mer (units). Annealing temperature depends on the number of G and C in the primer. i.e 2(A+T) + 4(G+C)= annealing temperature.

Primers Used For DNA Sequencing.

Primers used for sequencing *cnxH*.

F	CCA TCC TGT CAA GTC TTG.
F2	AAT GAC TGG AAG AGA GA
F3	ACC CAT CCC TCT CCC TG
F4	GCG ACG GGA CGG TCA TC
F5	TGC GTT GCG TTC GGA TC
F6	AAT AAC TGA CTG AAC TG
F7	GTA ACC AGC GTA CAT TG
F8	CGC CTC TTG AAC TCC AG
R	GGC AGT GCA GTT CAA GC
R2	GGA AGC CAA GGG AGG AA
R3	GAC GAG TAT GAG CAA GC
R4	TTG ATA TGC CAT TGA TC
R5	TCA AGC TTA TCA GTT CC
R6	CCA CCT CGA GCT AAC AT
R7	GAG GTT GTG AAG GAG CG

NB. primers R and F were used in order to get a PCR product to sequence *cnxH* mutants.

PCR Conditions.

I Primers Used For Clonning.

Primer Set 1 (Primers λ1 and λ2).

Cycles 1; Denaturing (94°C for 2 min), Annealing (50°C for 30 sec), DNA synthesis (72°C for 30 sec). Cycles 2-35; Denaturing (94°C for 20 sec); Annealing (50°C for 20 sec), DNA synthesis (72°C for 20 sec).

Primer Set 2 (Primers AMP 1 and AMP 2).

Cycles 1; Denaturing (94°C for 2 min), Annealing (50°C for 20 sec), DNA synthesis (72°C for 30 sec). Cycles 2-25; Denaturing (94°C for 20 sec); Annealing (50°C for 20 sec), DNA synthesis (72°C for 30 sec).

II Primers Used For DNA Sequencing.

Primer Set 3 primers used for sequencing *cnxH* (Primers R And F).

Cycles 1; Denaturing (94°C for 60 sec), Annealing (50°C for 15 sec), DNA synthesis (72°C for 50 sec). Cycles 2-31; Denaturing (94°C for 20 sec); Annealing (50°C for 15 sec), DNA synthesis (72°C for 50 sec).

2.4.2.2 Rapid Fungal DNA Preparation For Polymerase Chain Reaction.

a) 10 ml of glucose supplemented minimal medium with all reqiured supplements and 10 mM nitrate as sole nitrogen source, were inoculated with a loopful of *A. nidulans* transformant's conidia.

b) The culture was incubated at 37°C for 30 h , and 200 rpm.

c) Mycelium was harvested, and washed with sterile distilled water, and blotted on 3 MM chromatography paper, then placed in liqiud nitrogen (at least 100 mg wet weight of mycelium was obtained).

d) 200 μl of 10:50 TE solution were added to the frozen mycelium, and mixed gently (10:50 TE; 10 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.1% SDS, double autoclaved).

e) The suspension was incubated at 60°C for 10 min , freezed in liqiud nitrogen, and followed by incubation at 60°C for further 10 min. The suspension was vortexed and freezed again in liquid nitrogen, followed by incubation at 60°C for 10 min .

f) After incubation the suspension was centrifuged for 5 min at 14000 rpm.

g) The resulting supernatant was transferred to a further tube kept in ice. The DNA was precipitated with 0.1 vol NaOAC buffer, and two vol of 100% ethanol (previously stored at -20°C), then incubated at -20°C for 10 min.

h) DNA suspension was centrifuged for 10 min at 14000 rpm, and the pellet was washed with 70% cold ethanol, dried, and resuspended in 100 μl TE (10:1) by vortexing.

2.4.2.3 Polymerase Chain Reaction For Amplification Of *Aspergillus. nidulans* Cosmid, DNA Transformants (Using Primers λ1 And λ2).

PCR Reaction Mixture	Vol (μl)	Final Conc
a) Template DNA.	5	
b) 10 x dynazyme buffer.	10	1 x
c) 2 mM dNTP	5	100 μM
d) 140 μM primer λ1	0.7	1 μM
e) 170 μM primer λ2	0.6	1 μM
f) Enzyme.	0.5	
g) Sterile distilled water	78.2	

h) The total vol (100 μl) was overlaid with 2 drops of mineral oil, and the reaction was ran on programme 12 for 35 cycles.

i) After reaction completion 10 μl of the PCR product and 1 μl dye along with 0.5 μg/10 μl of100 base pair ladder (1:10), were checked by running on a 2% agarose gel with 1 X TAE buffer at 50 volts, for 2 h.

2.4.2.4 Polymerase Change Reaction For Amplification Of *Aspergillus. nidulans* DNA Transformants (Using Ampicillin Primers).

PCR Reaction

Mixture	Vol(μl)	Final Conc
a) Template DNA	5	
b) 10 x Dyna zyme buffer	10	1 x
c) 2 mM dNTP	5	100μM
d) 20 μM primer AMP1 (ampicillin1)	5	1 μM
e) 20 μM primer AMP2 (ampicillin 2)	5	1 μM
f) Dynazyme (mg-free)	0.5	5 μ/μl
15 mM $mgCl_2$	6	1.5 mM
g) sterile distilled water	63.5	

h) The total vol (100 μl) was overlaid with 2 drops of mineral oil, and the reaction was ran on programme 10 for 25 cycles.

i) After reaction completion 10 μl of the PCR product, and 1 μl of loading buffer, along with 0.5 μg /10 μl of 100 base pair ladder (1:10) were ckecked by running on a 2% agarose gel with 1 X TAE buffer, at 50 volts for 2 h..

2.4.3 DNA Sequencing.

DNA sequencing was carried out using the dideoxy-mediated chain termination method described by sanger *et. al.*, (1977). DNA was sequenced by means of a
sequenase version 2.0 kit (United States Biochemical Corporation).

Stock Solutions And Buffers.

Sequenase Buffer (5 X Concentrate).

200 mM Tris-HCl, pH 7.5
100 mM $MgCl_2$.
250 mM NaCl

Labeling Mixture (dGTP) (5 X Concentrate).

7.5 μM dGTp
7.5 μM dCTP
7.5 μM dTTP

Termination Mixture.

80 μM dGTP
80 μM dATP
80 μM dCTP
80 μM dTTP
8 μM ddGTP
50 mM NaCl.

Mn Buffer.

0.15 M sodium isocitrate, 0.1 M $MnCl_2$.

Stop Solution.

95% Formamide

20 mM Bromophenol blue

0.05% Xylene cyanol FF.

Enzyme Dilution Buffer.

10 mM Tris-HCl, pH 7.5

5 mM DTT

0.5 mg/ml BSA.

Sequenase DNA Polymerase Enzyme.

20 mM KPO4, pH 7.4.

1 mM DTT

0.1 mM EDTA

50% Glycerol.

Glycerol Enzyme Dilution Buffer.

20 mM Tris-HCl, pH 7.5

2 mM DTT

0.1 mM EDTA

50% Glycerol.

10 X TBE.

109.0 gm Tris-base
55.6 gm Boric acid
7.4 gm EDTA
Total Volume 1 L, pH 8.3.

2.4.3.1 DNA Preparation From Wild-type, Temperature Conditional And Non-Conditional *cnxH* Mutant Strains For Sequencing.

a) Genomic DNA was prepared using the nucleon II kit (ScotLab, U.K), (See fungal genomic DNA preparation using nucleon II kit).

b) Genomic DNA from wild-type and *cnxH* mutants (*cnxH427* , *cnxH911* and *cnxH261*) was digested by *EcoR* I (does not cut the gene),

Digestion Mixture	Vol (μl)
DNA	10
RNase	1
10 x buffer	10
EcoR I	3
H_2O	76

c) The total volume (100 μl) was incubated at 37°C for 3 h, 1/10 vol of NaOC, and 2 vol of 100% cold ethanol were added to the digested DNA, then incubated at -70°C for 15 min, followed by centrifugation at 14000 rpm for 10 min.

d) The DNA pellet was washed with 100 μl of 70% cold ethanol at -20°C and centrifuged for 10 min at 14000 rpm, dried and resuspended in 50 μl TE solution (10:0.1).

DNA Dialysis.

a) Digested DNA was dialysed for 1 h at room temperature in TE solution (10:0.1), using the millipore membrane (millipore type VS 0.025 mm).

b) 2 μl of the dialysed DNA were checked by running on a 0.8% agarose gel with 1 X TAE buffer at 50 volts for 1 h, in order to estimate the concentration.

2.4.3.2 Polymerase Chain Reaction (PCR) For Dialysed DNA.

PCR Reaction Mixture	Vol (μl/sample)
Dialysed DNA (100 ng)	2
10 x dynazyme buffer	10
100 μM foreward primer (F)	1
100 μM reverse primer (R)	1
10 μM dNTP	1
Dynazyme	0.5
double autoclaved distilled water	84.5

a) The total volume (100 μl) was overlaid with 2 drops of mineral oil, and the reaction was ran on programme 25.

b) After the PCR reaction completed 10 μl of PCR product and 1 μl of loading buffer, along with 0.5 μg/10 μl 100 bp ladder were checked by running on a 2% agarose gel, with 1 X TAE at 50 volts, for 2 h.

2.4.3.3 Isolation Of DNA Fragment From Agarose Gel Using Gene Clean Kit.(Bio 101, California, Product no. 1001-400)

a) 20 μl of PCR product were ran on an 0.8% agarose, with 1 X TAE, at 50 volts for 2 h.

b) The gel was placed over 4 layers of clingfilm, quickly the gel block containing the correct DNA fragment was cut and chopped in 1.5 ml Eppendorf tube.

c) 600 ml of NaI solution were added, and incubated at 50°C for 5 min to dissolve.

d) After incubation 10 ml of glassmilk (resuspended by vigorous vortexing just prior to use), were added to the DNA suspension and incubated at room temperature for 10 min.

e) DNA suspension was centrifuged for 20 sec, at 14000 rpm, and the pellet was washed 3 times each with 600 ml New wash solution (previously stored at -20°C). In each washing step the DNA pellet was resuspended by vortexing, then followed by centrifugation for 20 sec.

f) After the last wash, the DNA was centrifuged for further 20 sec, and all remaining liquid was removed by fine tip .

g) After centrifugation the DNA Pellet was resuspended in the same volume of SDW as the original glassmilk volume (10 μl), then eluted at 50°C for 5 min.

h) DNA Suspention was centrifuged for 20 sec, and the aqueous phase containing the DNA was removed using fine tip, whilst avoiding glassmilk pellet.

i) DNA concentration was estimated by running 1 μl along with known amount of 100 base pair ladder (0.5 μg/10 μl), on an 0.8% agarose gel with 1 X TAE buffer at 50 volts for 2 h.

2.4.3.4 Annealing Template And Primer.

a) For each set of 4 sequencing lanes, a single annealing (and subsequent labelling) reaction was used.

b) To a 9 μl of treated PCR DNA product , 1 μl of primer (10 pmol/μl) was added, and denatured by heating at 99°C for 3 min in the PCR machine.

c) After denaturing the DNA, the DNA tubes were transferred immediately to ice for 3 min, and centrifuged for 20 sec then incubated in ice.

2.4.3.5 Labelling Reaction.

The method used was according to Sambrook *et. al.*, (1989).

a) To an ice-cold annealed DNA mixture (10 μl) the following solutions were added.

Sequenase reaction buffer	2.0 μl
0.1 M DTT	1.0 μl
diluted labelling mixture - dGTP	2.0 μl
(stock reagent was diluted 1:5 with water to a concentration of 1.5 mM).	

^{35}S dATP 5 mci	0.5 μl
sequenase DNA polymerase (was diluted with sequenase dilution buffer 1:8)	2.0 μl

c) The labelling mixture was incubated at room temperature for 5 min .

2.4.3.6 Termination Reaction.

a) For each DNA sample 4 tubes were labelled as G, A, T, and C, 2.5 μl of the appropriate dideoxy termination mixture (ddG, ddC, ddT, and ddA) was added.

b) The tubes were pre-warmed to 37°C, and 3.5 μl of the labelling reaction mixture were added to each, then incubated at 37°C for 5 min.

c) After incubation 4 μl of stop solution were added to each termination reaction, mixed and incubated in ice.

d) Before loading the sequencing gel, reaction mixtures were heated at 75°C for 5 min, and 3 μl of each reaction mixture were loaded in each lane.

2.4.3.7 Preparation Of Sequencing Gel.

(Bio rad sequi-Gen Nucleic acid sequencing system)

a) Sequencing gel solutions, Easy Gel (ScotLab, product number.9222)

Stock Solution	Vol/80 ml
Urea	33.6 g
10 X TBE	08.0 ml
Easy Gel	12.0 ml
25% APS	
TEMED.	

Preparation Of Plates.

a) Sequencing Plates were cleaned by DECON solution, and rinsed well with water, then 70% ethanol.

b) The brick plate was siliconised in the fume cupbooard, washed and checked for radioactive contaminants.

Sealing Gel.

a) Two strips of 3 MM chromatography paper, were placed in the pouring support, and sealing gel solution was poured along the length of the paper. The gel rig was pressed firmly down into the support from right to left with tightening the screws and left for 1 h to solidify .

Sealing Gel	Large Rig (38 X 50)	Small Rig (21 X 50)
Gel solution	040 ml	020 ml
25% APS	200 μl	100 μl
TEMED	200 μl	100 μl.

Sequencing Gel.

Gel Mixture	Large Rig (38 X 50)	Small Rig (21 X 50)
Gel solution	110 ml	60 ml
25% APS	110 μl	60 μl
TEMED	110 μl	60 μl

a) using a large syringe, the sequencing gel mixture was poured at 30°C, and the spacer combs were inserted in inverted position, then left for at least 30 min, or overnight.

b) Before loading the reaction mixtures, prerunning the gel at 1600 volts to 1800 volts was necessary to warm the gel and the buffer to 50°C.

Loading And Running The Gel.

a) Immediately prior to loading, sequencing reactions were heated to 75°C for 2 min, and urea was washed from wells with 1 X TBE buffer using a 50 ml syringe.

b) 3 μl of each reaction were loaded in each lane, and the gel was run for 5 h until the dye front was at the end of the gel (a second loading and run for 2 h, was allowing further sequence determination).

Gel Drying.

a) After the completion of DNA sequencing the buffer was poured into approved disposal sink, and the rig was laid on the bench with reservoir side up.

b) The plates were separated and smooth 3 MM paper was placed over the gel.

c) A Sheet of 3 MM paper was placed on the dryer (BioRad, model 583), and the sequencing gel was placed on the top, the gel was covered with cling film and left to dry at 80°C for 2 h, .

d) The gel was checked for incorporation using the Gieger counter, and exposed to an X-ray film at room temperature.

2.5 Biochemical Assays.

2.5.1 Nitrate Uptake Assays.

The procedure used by Brownlee and Arst (1983), was followed in this research work.

Media.

Minimal and complete media described previously by Cove (1966) were used throughout. The minimal medium contained 10 g of D-glucose per litre (as a carbon source). The nitrogen sources were added at 5 mM (urea), or 10 mM (nitrate). Nitrate was added as sodium salt.

Growth Tests.

Strains were inoculated on solid complete media, and incubated at 37°C for 5 days.

Mycelia And Nitrate Uptake Assay.

a) Conidiospores were harvested from Petri dishes containing complete medium (Cove, 1966) into 10 ml sterile saline/Tween 80 solution (0.9% saline, 0.01% Tween 80), and were used for inoculation of liquid minimal media for growth of mycelia needed for nitrate uptake assays.

b) Approximately one-fifth plate (Brownlee and Arst, 1983) per 200 ml of liquid supplemented glucose, minimal medium (Cove, 1966) in one litre polypropylene Erlenmeyer flask was used. Supplements were added at standard levels (Cove, 1966). Urea as a sole nitrogen source was used at a final concentration of 5 mM. Mycelia were grown at 37°C or 25°C (as required) in a orbital shaker at 200 rpm for 8, 12 or 16 h.

c) 100 min before harvesting mycelia were washed with nitrogen free minimal media (incubated at the same temperature), and transferred to a further 200 ml minimal medium. Nitrate as sodium salt was added to the new media at a final concentration of 10 mM.

d) At harvesting time, mycelia were harvested through Mira-cloth, and washed as before.

e) Blotted wet weight mycelia (2 mg/ml) were transferred to 250 ml Erlenmeyer flasks containing 50 ml nitrogen free glucose minimal medium (pH 6.5). Supplements with (per litre) 10 mg of biotin, and

5 mg of pyridoxine hydrochloride (as necessary) were added to the media and incubated at 37°C in a receprical shaking water bath at 80 rpm.

NB: For each age of cells, the blotted wet weight of mycelia was transferred into 3 X 50 ml nitrogen free minimal medium, leaving two more flasks without mycelia as controls. Flask 1 (N^+: contains mycelia and nitrogen source). Flask 2 (N^-: contains mycelia but not nitrogen source). Flask 3 (Blank: mycelia free, nitrogen free). These flasks were used for measuring nitrate uptake values after 10 min, from the addition of nitrogen source. The same number of flasks, with the same constituents was used for measuring nitrate uptake values after 20 min from the addition of nitrogen source.

f) 500 μM sodium nitrate were added to the N^+ flasks.

g) Uptake values in terms of $nmol.min^{-1}.mg^{-1}$ blotted wet weight, were estimated after 10 and 20 min from the addition of the nitrogen source, by following the disappearance of nitrate from the media (Goldsmith *et. al.*, 1973; Schloemer and Garrett, 1974; Brownlee and Arst, 1983).

h) Nitrate concentration in the assay media was determined spectrophotometerically (SP6-550 UV/VIS PYE Unicam spectrophotometer) from the decrease in absorbance at 204 nm of acidified samples (suitably diluted in 5% perchloric acid). Values obtained were converted into amounts of nitrate by means of applying the absorbance into an equation obtained from nitrate standard curve (appendix 1) prepared along with the experiment.

i) Nitrate uptake values were based upon at least three rapidly filtered and acidified 3 ml samples taken after 10 or 20 min from the addition of the nitrogen source.

2.5.2 Nitrite Uptake Assays.

The procedure used by Galvan *et. al.*, (1996), has been followed in this research with minor modifications.

Media.

Minimal and complete media described previously by Cove (1966) were used throughout. The minimal medium contained 10 g D-glucose per litre (as a carbon source). Nitrogen sources were added at 5 mM (urea), 10 mM (nitrate), 10 mM (nitrite), or 10 mM (ammonium). Nitrate and nitrite were added as sodium salts, ammonium was added as ammonium tartarate.

Growth Tests.

Strains were inoculated on solid complete media, and incubated at 37°C for 5 days.

Mycelia.

Conidiospores (one fifth of a plate) were harvested from Petri dishes containing complete medium (Cove, 1966), and transferred into 10 ml sterile saline/Tween 80 solution (0.9% saline, 0.01% Tween 80). The suspension was vortexed properly and used for inoculation of liquid minimal medium for growth of mycelia needed

for nitrite uptake assays. Approximately one-fifth plate per 200 ml of liquid supplemented glucose minimal medium in one litre polypropylene Erlenmeyer flask. Supplements were added at standard levels (Cove, 1966). Urea as a sole nitrogen source was used at a final concentration of 5 mM. Mycelia were grown at 37°C in a orbital shaker at 200 rpm for 8 or 16 h. A 100 min or 3 h before harvesting (as required) and after washing the cells with nitrogen free minimal medium (see nitrate uptake assays) nitrate, nitrite, ammonium, or nitrate and ammonium were added at a final concentration of 10 mM. At harvesting time mycelia were harvested through Mira-cloth, and washed with nitrogen-free minimal medium (incubated at the same temperature as mycelia at the induction or reoression period) and used for transport experiments.

Nitrite Assay.

a) Blotted wet weight mycelia (1 mg /ml) were transferred to 250 ml Erlenmeyer flasks incubated at 37°C in a reciprocal shaking water bath, containing 50 ml nitrogen-free glucose minimal medium. The 50 ml nitrite assay media (pH 6.5) was supplemented with the following: 10 μg of biotin, 5 mg of pyridoxine hydrochloride (per litre, as necessary), 50 mM Tris-HCl buffer pH 7.5, 20 mM KCl, and mycelia (1 mg /ml).

b) After 5 min nitrite as sodium salt was added to the media from 10 μM up to 10000 μM (as required).

c) samples (at least three) were rapidly filtered every thirty seconds up to 2 min, then after every three min up to 21 min.

d) Nitrite concentration in the medium was measured colorimetrically according to (Snell and Snell, 1949; Gavan *et al.*, 1996). Three rapidly filtered samples each of 0.5 ml were transferred to clean dry small glass test tube, and 1 ml of 1% (w/v) sulphanilamide in 1:4 HCl was added to each tube followed by adding 1 ml of 0.02% (w/v) NED solution, the contents were mixed gently by hand.

e) The tubes were incubated at room temperature for 20 min, for colour development.

f) The resultant pink colour was proportional to the amount of nitrite present, and was estimated by determining the absorbance of the assay mixture at 540 nm spectrophotometrically (SP6-550 UV/VIS PYE Unicam spectrophotometer).

g) Values were converted into amounts of nitrite by means of applying the absorbance value to an equation obtained from a nitrite standard curve (appendix 2) prepared under the same conditions.

h) Control tubes lacking nitrite were used to correct for nitrite present in the media.

Kinetics Of Nitrite Uptake.

Kinetics of nitrite uptake was studied by using the following procedures:

a) Initial rate method.

Initial rate of nitrite uptake at different nitrite concentrations in the medium was estimated at pH (6.5) in diluted cell cultures (1 mg/ml), as the mean velocity over a short period (3 min), which is assumed to

be the initial velocity at the corresponding substrate concentration. Kinetic parameters were calculated using the ENZ FITTER programme devised for the IBM computer.

b) Analytical determinations.

nitrite uptake rates in terms of $nmol.min^{-1}.mg^{-1}$ blotted wet weight were determined at pH 6.5, by measuring nitrite disappearance from the medium at different times.

2.5.3 Nitrate Reductase Assays.

A slightly modified procedure from that described by (Wray and Filner, 1970; MacDonald and Cove, 1974) was used in this research.

Media.

Minimal and complete media described previously by Cove (1966) were used throughout. The minimal medium contained 10 g of D-glucose per litre as a carbon source, nitrogen source was added at 10 mM (nitrate) as sodium salt.

Growth Tests.

Strains were inoculated on solid complete medium and incubated at 37°C for 5 days.

Mycelia.

Conidiospores were harvested from Petri dishes containing complete medium (Cove 1966) and transferred into 20 ml sterile saline/Tween 80 solution (0.9% saline, 0.01% Tween 80). The suspension was vortexed properly and used for inoculation of liquid minimal medium for growth of mycelia. One plate per 400 ml of liquid supplemented glucose minimal medium (Cove, 1966) in one litre polypropylene Erlenmeyer-flask was used. The supplements were added at standard levels (Cove, 1966). Nitrate as sodium salt (10 mM) was added. Mycelia were grown at 25°C for 21 h in an orbital shaker at 200 rpm.

Harvest Of Mycelium.

Mycelium was harvested by filtering through Mira-cloth, and washed thoroughly with sterile distilled water, and finally blotted dry with absorbent paper. Mycelium was stored in liquid nitrogen for few min before starting the assay experiment.

Preparation Of Cell-Free Mycelial Extract.

a) Mycelium was ground by hand in a cold mortar (kept at 4°C overnight), together with 10 times its weight (0.5 g fresh weight/5 ml extraction buffer) of cold extraction buffer.

b) The extraction buffer was containing 100 mM Tris-HCl buffer (pH 8.5), 3 mM DTT, 5 mM FAD, 1 mM Na_2EDTA pH 8.5, 1 mM sodium molybdate (made up fresh immediately before use).

c) The crude homogenate was centrifuged at 4°C at 16000 rpm for 20 min, and 0.1 ml of extract was incubated in ice bath, whilst the remaining vol was incubated at 35°C.

d) At timed intervals of 3 min up to 21 min aliquots of 0.4 ml were transferred to small glass test tubes incubated in ice. In the assay system 6 test tubes were arranged for each time interval (3 tests and 3 controls).

Enzyme Assay.

a) An assay mixture for each test tube in the assay system was made as described by Wray and Filner (1970) with slight modification as follows. 0.5 ml of 0.1 M potassium orthophosphate buffer pH 7.5, 0.1 ml of 0.1 M KNO_3, 0.1 ml of 1 mM NADPH, 0.25 ml of sterile distilled water.

b) The tubes containing this assay mixture were incubated at 25°C for 5 min in a water bath (blank tubes were NADPH-free) for temperature equilibration.

c) At timed intervals of 40 seconds 50 μl of crude extract from each incubation period were transferred from ice to each assay mixture tube. The assay mixture with the extract were incubated in a water bath at 25°C for 20 min.

d) After the 20 min incubation and according to the same timed intervals (40 sec), the reaction was stopped by adding 100 μl of ZnAc and 100 μl of PMS solution to each tube in the assay system.

e) 1 ml of 1% (w/v) sulphanilamide in 3 M-HCl, and 1 ml of 0.02% (w/v) NED solution were added next.

f) The contents of each tube were mixed gently by hand, and the precipitated protein was sedimented in a bench centrifuge for 5 min at 4500 rpm.

g) The tubes were incubated at room temperature for 20 min for colour development.

h) The resultant colour was proportional to the amount of nitrite present, and was estimated by determining the absorbance of the assay mixture at 540 nm spectrophotometrically (SP6-550 UV/VIS PYE Unicam spectrophotometer).

i) Values were converted into amounts of nitrite by means of applying the absorbance value to an equation obtained from a nitrite standard curve (appendix 2). Control tubes lacking NADPH were used to correct for nitrite present in the extract, and any turbidity caused by the sample.

Protein Determination.

a) The amount of soluble protein in the extract was estimated by using the Bio-Rad protein standard II (lyophilised BSA).

b) The crude extract was diluted 1 in 3 with the extraction buffer.

c) 100 μl of the diluted extract was transferred to each test tube in the assay system (control tubes were containing 100 μl of extraction buffer instead of crude extract).

d) 5 ml of the Bio-Rad diluted dye-reagent were added to each test tube, mixed by gentle shaking to avoid foaming and incubated at room temperature for 15 min.

e) Protein content in the extract was estimated by measuring the absorbance at 595 nm (SP6-550 UV/VIS PYE Unicam spectrophotometer) (water was used to zero the spectrophotometer).

f) Using a programme devised for apple Macintosh computer (CA cricket graph III, version 1.5) a protein standard curve was prepared where in each time the assay was performed a protein standard curve was prepared (representative protein standard curves for *crn*, *gdh-niaD* transformants, or the temperature-sensitive *cnx* mutants are presented in appendices 3 to 8).

g) The determined absorbance was applied to a equation obtained from the standard curve, inorder to determine the protein concentration in the extract in mg protein /ml crude extract.

h) Units of enzyme activity obtained per ml extract were divided by the protein concentration in that sample in order to calculate the enzyme specific activity in terms of units per min per mg protein.

Measurement Of The Half-Life Of *Aspergillus. nidulans* Nitrate Reductase.

The half-life of the enzyme nitrate reductase was estimated using the previously mentioned CA-cricket graph programme. The specific activities of the enzyme were calculated in terms of relative activities (specific activities normalised to the maximum specific activity at time = 0). The half-lives were estimated after determining the 50% activity of the enzyme.

2.6 Source Of Materials.

All chemicals used in this study were pure biochemical or AnalaR grade, obtained from either BDH or Sigma Chemical Co. Fungal media were from Amersham or Fluka. Bacterial media were from GIBCO BRL, or DIFCO laboratories. Restriction enzymes and their relevant buffers were from Pharmacia or GIBCO . BRL. Novozyme-234, batch number 1961 and 1906 were supplied by NovoBioLabs. Qaigen-columns were supplied by Qaigen. NucleonII kit for genomic DNA preparation was from ScotLab. Other kits, membranes for hybridisation were purchased from Amersham and MSI. Primers for PCR reactions and sequencing were either made in the University of St.Andrews or purchased from Amersham. Autoradiographic films were supplied by Kodak, and μ32-dCTP was obtained from ICN.

2.7 Containment And Safety.

All experimental procedures in this work were carried out with reference to the health and safety handbook, for the School of Biological and Medical Sciences at the University of St. Andrews. All work carried out was done within the guidance notes for safe use of display screen equipment, in the enviromental, health and safety services at the University of St. Andrews.

CHAPTER THREE

ISOLATION AND GROWTH CHARACTERISATION OF CHLORATE RESISTANT MUTANTS.

3.1 The Objectives Of This Research Section.

There were two main aims, first to generate many more *crn* mutants, and second to synthesis *cnx* mutants.

Strains Used.

Wild-type strains, with respect to nitrate assimilatory genes, were used in a number of experiments similar to that in previous reported work by Cove (reviewed by Cove, 1979). In such wild-type strains, the *niaD* gene, encoding nitrate reductase, is repressed in ammonium or glutamate grown cells, a phenomena known as nitrogen metbolite repression (Arst and Cove, 1973). In this study and as before, wild-type strains for nitrate assimilation were used to generate mutants. Additionally, genetically engineered transformants were also used for chlorate resistant mutant isolation. In these transformants the *niaD* gene was not subject to nitrogen metbolite repression. Such strains were used in anticipation that new genes might be revealed further to the ones identified. For a review see Cove, 1979 and references therein.

Isolation Of *crn* Mutants.

Whilst much detailed information is available regarding the nitrate assimilatory enzymatic steps including their structure, function, and regulation much less is known about the transport systems involved. Additionally, previous results from biochemical and molecular studies suggested the existence of more than one transport system. The aim of this particular study was to isolate a very large number of chlorate resistant mutants with a *crn* phenotype (i.e chlorate resistant, nitrate utilising) exploring therefore the possibility of unearthing further nitrate transport genes through a mutant isolation and sexual analysis between previously isolated *crnA* mutants and the *crn* mutants isolated during this study.

Isolation Of *cnx* Mutants.

For *cnx* (molybdenum cofactor required for nitrate reductase and purine hydroxylase activities) genes, the properties of the temperature-sensitive mutations should provide valuable evidence regarding the role of the *A. nidulans* *cnx* genes in the nitrate reductase and purine hydroxylase activities. Since some of the previously isolated (Cove, 1974) temperature-sensitive *cnx* mutants have been lost or at least not made available to us, the aim due was to identify new temperature-sensitive mutations in the *cnx* genes. Through the protein sequence analysis of a particular temperature-sensitive mutants along with the wild-type sequence of that particular gene the role of that *cnx* gene products in the nitrate assimilation pathway may be determined.

3.2 Mutagenesis.

Two wild-type strains (G1 *biA1* and A220 *yA2 pyroA4*), three [*gdhA-niaD*] transformant strains (SAA1040, SAA1032, SAA1023a), and one [*gdhB-niaD*] transformant strain (SAA1023b) (for full genotypes see section 2.1) were used in this study. Such strains were used untreated (i.e spontaneous mutants) or after a chemical mutagenic treatment (with either NTG or DEO). Mutants were selected at two temperatures (25°C or 37°C) (for details see section 2.3.1.6). Chlorate resistant micro-colonies developed (above the general growth background) after incubation at the selection temperature. These were taken and inoculated into chlorate containing master plates (25 mutants per master plate) of glucose supplemented minimal medium, pH 6.5, having the same constituents as the selection medium (see materials and methods section) and incubated at the selection temperature. Finally the resistant mutants were further inoculated onto a chlorate master plate (i.e a second master plate) having the same constituents as the first master plate, and incubated at the selection temperature (again 25 mutants per master plate). This latter was adopted as a further purification step.

3.3 Growth Tests.

Chlorate resistant mutants were replicated onto three different types of glucose supplemented minimal media, pH 6.5 containing the following: (1) 300 mM potassium chlorate (and a nitrogen source) at a concentration equivalent to what has been used with the original selection. (2) Minimal medium with adenine (5 mM) as the sole

nitrogen source. (3) minimal medium with nitrate (5 mM) as the sole nitrogen source. Two series of plates were inoculated in this way, one set was incubated at 25°C, and one set at 37°C.

The mutants isolated on the bases of chlorate resistance were phenotypically classified into *cnx*, *crn*, and *niaD* or *nirA* mutants according to growth criteria listed in Table 3.1. For example chlorate resistant mutants which showed growth on both nitrate and adenine were classified as *crn* mutants. Any mutant which lacked growth on both nitrate and adenine was classified as *cnx* mutant. Mutants which showed growth only on adenine were classified as *niaD* or *nirA* mutants (These were unwanted and therefore descarded for the purpose of this study). Analysis of all series of mutant selection experiments (Table 3.2) confirmed that from a total of 12,628 screened mutants 11,807 (approx 94% of the original screened mutants) were indeed confirmed chlorate resistant mutants. Of these 2082 (approx 18% of the total chlorate resistant mutants) were phenotypically *cnx* mutants, 425 (approx 4% of the total chlorate resistant mutants) *crn* mutants, and 9300 were *niaD* or *nirA* mutants. Table 3.2 shows the total number of each group of mutant selection, in addition to the treatment, nitrogen source, selection temperature and the treated strain used in the mutagenesis.

3.4 Factors Influencing The Ratio Of Generated Mutants.

3.4.1 The Influence Of proline As A Sole Nitrogen Source On The Ratio Of Chlorate Resistant Mutants Generated After Spontaneous Or NTG Treatment Of Wild-Type Strains.

The results presented in Table 3.2 showed that when proline was used as the sole nitrogen source after spontaneous mutagenesis of wild-type strains (i.e.*biA1* or *yA2 pyroA4*), *cnx* mutants comprised 35% of the total chlorate resistant mutants selected at 25°C, compared to 6% at 37°C. On the other hand, *crn* mutants comprised 25% when selected at 25°C compared to 10% when selected at 37°C. Furthermore, when proline served as the sole nitrogen source with wild-type strains after NTG treatment, *cnx* mutants were found to be 14% of the total chlorate resistant mutants, selected at 25°C, compared to 2% when selected at 37°C. Whilst selected *crn* mutants were present at a level of 4% at 25°C, and they were present at 9% when selected at 37°C.

These findings indicate that the highest percentage of chlorate resistant *cnx* and *crn* mutants was obtained after spontaneous rather than NTG mutagenesis of wild-type strains, especially when proline served as the sole nitrogen source at 25°C. These results confirm not unexpectedly the Cove's original observation that both the nitrogen source and the selection temperature influences the ratio of selected mutants.

3.4.2 The Influence Of Uric Acid As A Sole Nitrogen Source On The Ratio Of Chlorate Resistant Mutants Generated After Spontaneous, NTG Or DEO Treatment Of Wild-Type Strains.

No *cnx* mutants were selected at either 25°C or 37°C when uric acid served as the sole nitrogen source for wild-type strains after spontaneously mutagenesis. Furthermore, 8% of *crn* mutants were selected at 25°C whilst 18% were selected at 37°C. In addition, when uric acid served as the sole nitrogen source, the percentage of *cnx* mutants obtained at 25°C after NTG treatment was found to be 16% compared to 5% at 37°C. Whilst 10% *crn* mutants were obtained at 25°C, 25% were obtained at 37°C. These findings showed that *cnx* mutants are less frequent than *niaD*, *nirA*, or *crn* mutants when uric acid served as the sole nitrogen source, but more frequent when the mutagen NTG or DEO treatment was applied at both selection temperatures. These results lead to the conclusion that the nitrogen source used with the treatment has an obvious influence on the ratio of the genotypes of mutants selected.

The percentage of *cnx* mutants obtained after DEO chemical mutagenesis treatment of wild-type strain (*biA1*) when uric acid was used as the sole nitrogen source was 17% at both selection temperatures, whereas *crn* mutants were found at 1% again at both selection temperatures. These data indicate that the DEO treatment was the one of choice for selecting a high percentage of *cnx* mutants in this case at least where uric acid was acting as the sole nitrogen

source for growth of wild-type whatever the selection temperature (i.e.either 25°C or 37°C). NTG treatment was better than DEO and spontaneous treatments for obtaining *crn* mutants at 25°C selection temperature. These data indicate the influence of nitrogen source, the selection temperature, and the kind of treatment on the ratio of the type of selected mutants.

3.4.3 The Influence Of Glutamate As A Sole Nitrogen Source On The Ratio Of Chlorate Resistant Mutants Generated After Spontaneous, NTG Or DEO Treatment Of Wild-Type Strains.

After spontaneous treatment and when glutamate was used as the sole nitrogen source with wild-type strains (*biA1* or *yA2 pyroA4*), selection at 25°C gave 1% *cnx* and 6% *crn* mutants. After NTG treatment, 10% *cnx* mutants were obtained at 25°C, and 2% at 37°C. In addition 8% *crn* mutants were obtained at 25°C but, no *crn* mutants were obtained at 37°C. After DEO treatment, 9% *cnx* mutants were generated at 25°C, and 6% at 37°C, whilst, 22% *crn* mutants were observed at 25°C and 10% at 37°C. These data indicate that, for the purpose of generating either *cnx* or *crn* mutants i.e. the objective in this study, glutamate was not the nitrogen source of choice whatever the mutagenic treatment.

3.4.4 The Influence Of The *gdhA-niaD* Transformant Strain SAA1040 On The Ratio Of Chlorate Resistant Mutants Generated After DEO Treatment.

All *gdhA-niaD* transformants (SAA1040, SAA1032, and SAA1023a) and *gdhB-niaD* transformant (SAA1023b) (for full genotypes of these strains see section 2.1) were treated with the mutagen DEO and selected at 37°C. When proline served as the sole nitrogen source transformant strain SAA1040, yielded *cnx* mutants at only 2% of the confirmed chlorate resistant mutants, this is compared to 3% and 31% when ammonium and urea, respectively, were used as the nitrogen source. These data indicate that urea was the best nitrogen source for the generation of *cnx* mutants with strain SAA1040.

3.4.5 The Influence Of The *gdhA-niaD* Transformant Strain SAA1032 On The Ratio Of Chlorate Resistant Mutants Generated After DEO Treatment.

The percentage of *cnx* mutants obtained when proline served as the sole nitrogen source with the transformant strain SAA1032 was 10% compared to 20% and 2% with ammonium and urea, respectively. This indicated that ammonium was the best nitrogen source for generating *cnx* mutants with this transformant strain.

3.4.6 The Influence Of The *gdhA-niaD* Transformant Strain SAA1023a On The Ratio Of Chlorate Resistant Mutants Generated After DEO Treatment.

In contrast, no *cnx* mutants were selected when either ammonium or urea were used as sole nitrogen sources with the transformant SAA1023a, strain whereas, only 2% *cnx* mutants were generated when proline was used as sole source of nitrogen. Such results show the influence of the treated strain on the ratio of selected mutants.

3.4.7 The Influence Of The *gdhB-niaD* Transformant Strain SAA1023b On The Ratio Of Chlorate Resistant Mutants Generated After DEO Treatment.

Surprisingly, when either proline or ammonium were used as sole nitrogen source with the transformant SAA1023b the percentage of *cnx* mutants obtained was over 52% of the confirmed chlorate resistant mutants.

3.5 Nitrate Reductase Activity In *gdhA-niaD* And *gdhB-niaD* Transformant Strains.

Wild-type is sensitive to chlorate when any nitrogen source other than ammonium is used. This is related to the fact that ammonium represses the enzyme nitrate reductase, and chlorate can not be converted into the toxic form chlorite (see section 1.7: other genes which may be required for nitrate assimilation for a discussion on chlorate resistance). The *gdh* transformants used in this study

(see section 2.1: strains used for mutagenesis) have a *gdh* promoter fused to the *niaD* gene (*gdhA* and *gdhB* are the structural genes for the enzymes NADP and NAD-glutamate dehydrogenase respectively). This fusion was inserted into a *argB* containing plasmid, that has the *argB* mutant. Consequently, this gene fusion (ie. *gdh-niaD*) after transformation should be integrated at the *argB* locus usually by a single copy. The *gdh* promoter is stimulated by ammonium in the medium, and can express *niaD* nitrate reductase even in the presence of ammonium (see nitrate reductase results section). Therefore transformant strains are sensitive to chlorate even with ammonium as the sole nitrogen source. By mutagenising such kind of transformants, the hope was to generate mutant(s) resistant to chlorate in the presence of ammonium by looking for mutations at another locus. This latter locus (gene) controls the *gdh* promoter, through which it will represses the production of nitrate reductase enzyme. This is called Trans-acting gene (gene controls another gene at another locus), so no toxic forms of chlorate will be formed, and this means the mutant becomes resistant to chlorate.

Nitrate reductase levels in wild-type *niaD* strain and *gdhA* or *gdhB* regulated *niaD* transformants were determined. Nitrate reductase activities in Table 3.3 showed that all genetically engineered transformant strains grown on nitrate had approximately wild-type *niaD* nitrate reductase activities, with the exception in transformant SAA1040 where enzyme levels were double as that of wild-type. However, all these transformants showed high levels of enzyme

activity when grown on ammonium as compared to wild-type (i.e undetectable) under the same growth conditions. In transformant strain SAA1040 nitrate reductase levels on ammonium were slightly higher than that on nitrate, and with two and a half folds higher than that of wild-type on nitrate. In transformant SAA1023a therewas no or little difference in enzyme levels between cells grown on nitrate and others on ammonium, where these levels were approximately the same as wild-type enzyme levels on nitrate, and approximately 50% lower than that of SAA1040 strain. In transformant SAA1023b enzyme levels on ammonium were reduced approximately 50% as compared to that on nitrate (i.e wild-type levels on nitrate) and 3 to 4 folds reduction as compared to SAA1040 strain. However, enzyme level on ammonium in SAA1023b strain was reduced 2 folds as compared to that on nitrate and 10 to 12 folds as compared to that of SAA1040. Data obtained indicate that *gdh-niaD* gene fusion transformants are no more controlled by nitrate, completely not regulated by nitrate and not repressed by ammonium. Also not nitrate induced either.

3.6 Comparison Study.

Cove (1976 a) has treated five different strains (*yA1 puA2*, *puA2*, *biA1*, *anA1yA1*, *biA1 puA2 pyroA4*) either spontaneously or with the mutagen NTG and selected at 37°C, in order to generate different classes of chlorate resistant mutants. The results are shown in Table 3.4. A comparison study between the results of this research section and that obtained previously (Cove, 1976 a) showed that,

when Cove treated the above wild-type strains spontaneously, arginine was not a good nitrogen source for the selection of either *cnx* or *crn* mutants. The percentage of *cnx* mutants obtained with arginine in the his work was within the range of 0.5% to 9%, while for *crn* mutants between 0% to 0.5%. These data indicate from the first instance, whatever the treated strain is, arginine as a sole nitrogen source was not useful for mutant selection at least after spontaneous treatment. In contrast, when Cove has changed the treatment instead using chemical mutagen (i.e NTG) surprisingly, the proportion of *cnx* mutants was increased to between 41% and 46%. However, only 0% to 12% *crn* mutants were generated after NTG treatment. These data indicate that the mutagenic treatment (i.e NTG) has an obvious influence on the ratio of *cnx* but not *crn* mutants. In our research section the idea was to change this nitrogen source (i.e arginine) hoping to generate both *crn* and *cnx* mutants at a higher ratio than that obtained previously. Unfortunately, when proline was used as sole nitrogen source with wild-type strains with selection at 37°C after spontaneous treatment only 10% *crn* mutants and 6% *cnx* mutants was obtained. When the chemical mutagenesis treatment was used (NTG) not surprisingly perhaps, only 9% *crn* mutants and 2% *cnx* mutants were generated. When the selection temperature reduced to 25°C, with proline and after NTG treatment 4% *crn* mutants and 14% *cnx* mutants was observed. In contrast, spontaneous treatment yielded 26% *crn* and 35% *cnx* mutants when proline was used as the sole nitrogen source. This indicates that, in addition, to the treated strain both the nitrogen source and the treatment were influencing the ratio of generated mutants.

When Cove (1976 a) used glutamate as sole nitrogen source, the level of selected *crn* mutants, after spontaneous treatment, was found to be 0% for *crn* and 4% for *cnx* mutants. In the current study the idea was to avoid repeating this particular treatment (i.e spontaneous) and to apply a mutagenic treatment instead. Not surprisingly, when a NTG treatment was applied to wild-type strains, the percentage of generated mutants was 0% for *crn* and 2% for *cnx* mutants. When the DEO treatment was used *crn* mutants formed 10% of the confirmed chlorate resistant mutants, while *cnx* mutants were forming 6%. The only left possibility was to change the selection temperature to 25°C, thus when glutamate was used with the wild-type strains after spontaneous treatment, glutamate was yielding 6% *crn* mutants and 1% *cnx* mutants. After NTG treatment the yield was 8% *crn* and 10% *cnx* mutants. However, after DEO treatment *crn* mutants were forming 22%, while *cnx* mutants were forming 9% of confirmed chlorate resistant mutants. These data indicate that glutamate was not acting as the best sole nitrogen source for mutant selection (specifically *crn* and *cnx* mutants) what ever the treatment, the treated strain, or the selection temperature was.

When Cove (after spontaneous treatment) has used uric acid as the sole nitrogen source with wild-type strains, selection at 37°C 0.5% to 6% *crn* mutants and 2% to 15% *cnx* mutants was observed. However, after NTG treatment, uric acid selection yielded 17% to 20% of *cnx* or *crn* mutants. In this section of this current research,

when wild-type strains were treated spontaneously, uric acid yielded 22% *crn* mutants, with no *cnx* mutants. Additionally, after NTG treatment uric acid treatment yielded 25% *crn* mutants which broadly agrees with Cove's findings, but, only 5% *cnx* mutants were generated with this treatment compared to 17% to 20% obtained in the Cove's previous work. Moreover, when DEO treament was applied with uric acid only 1% *crn* and 17% *cnx* mutants was observed. These data indicate that, at 37°C uric acid served as the sole nitrogen source for generating *crn* (but not *cnx* mutants), after spontaneous or NTG but not DEO treatment (of wild-type strains). In contrast, this nitrogen source yielded *cnx* but not *crn* mutants after DEO treatment of the wild-type strains at 37°C. When the selection temperature was changed to 25°C, with uric acid as a sole nitrogen source, it was not suitable for generating *crn* mutants, whatever the treatment was (i.e spontaneous, NTG, or DEO). In contrast, this nitrogen (i.e uric acid) selection treatment gave *cnx* mutants after NTG or DEO but none following spontaneous treatment of the wild-type strains.

3.7 Discussion.

Results of this current research section lead to the following conclusion:

First, proline as a sole nitrogen source for growth of wild-type strains was the best for generating chlorate resistant *cnx* and *crn* mutants when selected at 25°C after spontaneous rather than NTG treatment. Second, no *cnx* mutants were generated at both selection

temperatures (i.e 25°C and 37°C) when uric acid was the sole nitrogen source with wild-type strains (*biA1* and *yA2 pyroA4*) treated spontaneously. Third, uric acid was acting as suitable nitrogen source for selecting *crn* but not *cnx* mutants at 37°C after either spontaneous or NTG but not DEO treatment of the wild-type strains (*biA1* and *yA2 pyroA4*). In contrast, uric acid can serve at 37°C for generating *cnx* but not *crn* mutants after DEO treatment of wild-type strains. Fourth, glutamate was not a suitable nitrogen source for the generation of *cnx* mutants at either 25°C or 37°C when wild-type strains were treated with any of the three different treatments (i.e spont, NTG, or DEO). Fifth, glutamate might act as sole nitrogen source for generating *crn* mutants at 25°C after DEO treatment of the wild-type strains. Sixth, after DEO treatment transformant strain SAA1040 was suitable for selecting *cnx* mutants at 37°C when urea but not proline or ammonium was used as sole nitrogen source. Seventh, after DEO treatment, transformant strain SAA1032 was suitable for generating *cnx* mutants at 37°C when ammonium but not proline or urea was serving as the sole nitrogen source. Eighth, transformant strain SAA1023a after DEO treatment was not suitable for selection of *cnx* or *crn* mutants at 37°C when either ammonium, proline, or urea was used as the sole nitrogen source. Ninth, transformant SAA1023b was the best *gdh-niaD* transformant for generating *cnx* mutants at 37°C after DEO treatment when either proline or ammonium was used as the sole nitrogen source. Tenth, not surprisingly, perhaps, none of the *gdh-niaD* transformants was able to generate a single *crn* mutant at 37°C after DEO treatment.

Finally, these results provide strong evidences that the ratio obtained between *cnx* , *crn*, and *niaD* or *nirA* mutants has been obviously influenced by the following factors: First, the treatment used whether spontaneous or mutagenic treatment. Second, the nitrogen source used with the treated strain. Third, the treated strain itself. Fourth, the temperature at which the treated strain was selected at.

The results of this current research section show that in wild-type as the high relative frequency of *niaD* or *nirA* mutants amongst mutants obtained (*cnx* and *crn* mutants) might be explained as if the *niaD* or *nirA* mutants were more sensitive to either spontaneous or mutagenic treatment than other kinds of mutants. Additionally the results indicate that the rate of occurrence of all types of chlorate resistant mutants was however, higher if a mutagenic treatment was applied than if it was omitted. Furthermore, *cnx* mutants were found to be much commoner relative to *crn* mutants amongst chlorate resistant mutants selected after a mutagenic treatment than after spontaneous treatment. Not surprisingly perhaps, no single *crn* mutant was selected after DEO treatment with either proline, ammonium, or urea which were used as sole nitrogen sources with the transformants. These data suggest that *crn* mutants were more susceptible to DEO treatment than *cnx* or *niaD* or *nirA* mutants. This indication might be explained as if *cnx* genes are more sensitive to the mutagenic treatment than *crn* gene(s).

Table 3.1. Growth Criteria Used For The Classification Of Chlorate resistant Strains.

Mutant	Nitrate	Adenine	Resistance To Chlorate With The Following Nitrogen Sources				
			Pro	Glu	U.A	NH4+	Urea
cnx	-	-	R	R	R	R	R
crn	+	+	R	R	R	R	R
niaD or *nirA*	-	+	R	R	R	R	R
biA1, cnxH4 *	-	-	R	R	R	R	R
biA1 *	+	+	S	S	S	S	S

Mutants were tested at 25°C and 37°C on glucose supplemented minimal medium (supplemented with vitamins), pH 6.5, containing nitrate (5 mM), or adenine (5 mM) as the sole nitrogen source. Potassium chlorate (300 mM) with different nitrogen sources as indicated above, proline (10 mM), glutamate (10 mM), uric acid (10 mM), ammonium (10 mM) and urea (5 mM). R: denotes resistant, S: denotes sensitive, symbol (-): denotes no growth, symbol (+): denotes wild-type level of growth. symbol (*): denotes control strains.

Table 3.2. Isolation And Growth Characterisatation Of Chlorate Resistant Mutants Generated By Either Spontanous Or Induced Mutagenesis.

a
Two wild-type with regard to nitrate assimilation strains *biA1* (strain number G1) and *yA2 pyroA4* (strain number A220) were used in the mutagenesis.
b
The wild-type *biA1* strain was used in this treatment.
c
See section 2.1.1: strains used for mutagenesis.
d
Mutagenised strains were taken and inoculated into chlorate master plates (glucose supplemented minimal medium, pH 6.5 with potassium chlorate (300 mM) and proline (10 mM) as a sole nitrogen source) and incubated at the selection temperature.
e
Confirmed chlorate resistant mutants were tested at both temperatures (25° C and 37° C) at least three times on glucose supplemented minimal medium, pH 6.5 with: (1) potassium chlorate (300 mM) and proline (10 mM). (2) hypoxanthine (5 mM). (3) sodium nitrate (5 mM).Mutants were classified according to the criteria shown in Table 3.1.
f
The percentage of each mutant group (ie. *cnx*, *crn*, and *niaD* or *nirA* mutants) has been presented as a percentage of the total chlorate confirmed resistant mutants, within each specific selection temperature as indicated.

-Growth tests were carried out at the original selection temperature. For putative thermo- and cryo-sensitive mutants analysis was repeated at least three times at both temperatures (25° C and 37° C).
No: denotes number of mutants, symbol %: denotes percentage of the total confirmed mutants.

Treatment		Spontaneous					N-methyl-N-nitro-nitrosoguanidine (NTG)					
Strain		wild-type[a]					wild-type[a]					
N-Source		Proline		Glutamate	Uric acid		Proline		Glutamate		Uric acid	
Selection temperature		25°C	37°C	25°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C
Original screened mutants[d]		292	260	338	42	100	1453	416	1331	670	368	434
Confirmed chlorate resistant mutants[e]		267	253	282	36	82	1282	340	1229	650	197	338
cnx mutants	No	94	15	4	0	0	178	6	128	14	32	17
	%[f]	35.2%	5.9%	1.4%	0%	0%	13.9%	1.8%	10.4%	2.2	16.2%	5%
crn mutants	No	68	26	16	3	18	50	31	95	0	19	86
	%[f]	25.5%	10.3%	5.7%	8.3%	21.9	3.9%	9.1%	7.7%	0%	9.6%	25.4%
niaD or *nirA* mutants	No	105	212	262	33	64	1054	303	1006	636	146	235
	%[f]	39.3%	83.7%	92.9%	91.7%	75%	82.2%	89.1%	81.8%	97.8%	74.1%	69.5%

Table 3.2 Continued.

Treatment		1,2,7,8-Diepoxyoctane (DEO)									
Strain		wild-type[b]				Transformant SAA1040[c]			Transformant SAA1032[c]		
N-Source		Glutamate		Uric acid		Proline	Ammonium	Urea	Proline	Ammonium	Urea
Selection temperature		25°C	37°C	25°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C
screened mutants [d]		52	78	114	135	350	600	2705	300	425	250
Chlorate Resistant mutants [e]		23	52	113	130	350	600	2693	300	425	250
cnx mutants	No	2	3	22	22	6	21	836	31	84	4
	%[f]	8.7%	5.8%	16.9%	16.9%	1.7%	3.5%	30.9%	10%	19.8%	1.6%
crn mutants	No	5	5	2	1	0	0	0	0	0	0
	%[f]	22%	10%	2%	1%	0%					
niaD or *nirA* mutants	No	16	44	89	107	344	579	1857	269	341	246
	%[f]	70%	85%	79%	82%	98%	97%	69%	90%	80.2%	98%

Table 3.2 Continued.

Treatment	1,2,7,8-Diepoxyoctane (DEO)				
Strain	Transformant SAA1023a[c]			Transformant SAA1023b[c]	
N-Source	Proline	Ammonium	Urea	Proline	Ammonium
Selection temperature	37°C	37°C	37°C	37°C	37°C
Screened mutants[d]	675	125	75	598	442
Chlorate resistant mutants[e]	675	125	75	598	442
cnx mutants No[f]	15	0	0	318	230
cnx mutants %[f]	2.2%	0%	0%	53%	52%
crn mutants	0	0	0	0	0
niaD or *nirA* mutants No[f]	660	125	75	280	212
niaD or *nirA* mutants %[f]	98%	100%	100%	47%	48%

Table 3.3 Nitrate Reductase Activities In The Wild-Type *niaD* Strain And *gdhA* Or *gdhB* Regulated *niaD* Transformants.

Strain[a]	N-source (10mM)	Nitrate reductase activity[c,d]
Wild-type	NH_4^+	undetectable
	NO_3^-	64.60 ± 2.3
SAA1040[b]	NH_4^+	148.20 ± 9.7
	NO_3^-	121.52 ± 3.4
SAA1032[b]	NH_4^+	21.54 ± 8.0
	NO_3^-	63.04 ± 3.3
SAA1023a[b]	NH_4^+	69.83 ± 6.0
	NO_3^-	71.06 ± 4.3
SAA1023b[b]	NH_4^+	36.66 ± 1.4
	NO_3^-	63.29 ± 6.5

(a) Mycelia were grown in supplemented glucose minimal medium with nitrate or ammonium (10 mM) at 37°C for 12 h. **(b)** SAA1040: *yA2 methH2 niaD53 argB* +, SAA1032: *yA3 wA3 methH2 niaD4 argB* +, SAA1023a: *yA2 wA1 methH2 niaD18 argB* +, SAA1023b: *yA2 wA1 methH2 niiaD18 argB* + **(c)** Enzyme activities are given as nanomoles of NADPH oxidised per minute per milligram protein. Values are mean ± standard deviation (SD) of three independent grow up experiments. Assays were carried out at 25°C, pH 6.5. **(d)** Strains mentioned above were used for the isolation of *cnx* and *crn* mutants.

Table 3.4. Proportions Of Various Classes Of Chlorate Resistant Mutants Obtained In This Study And In Previous Studies.

Source of sample	Strain[a]	N-source[b]	Treat[c]	*niaD* or *nirA*	*crn*	Total *cnx*	Genetically analysed *cnx*	ABC	E	F	G	H
This study	wild-type strains G1 and A220	Pro	Spont	317	94	109	31	13	4	9	4	1
		Glu	Spont	262	16	5	5	2	0	0	3	0
		U.A	Spont	97	21	0	0	0	0	0	0	0
		Pro	NTG	1357	81	184	65	30	3	15	14	3
		Glu	NTG	1642	95	142	78	39	2	23	9	5
		U.A	NTG	381	105	49	46	17	1	14	11	4
	G1	Glu	DEO	60	10	5	5	3	0	1	1	0
		U.A	DEO	196	3	44	33	24	1	6	1	1
	SAA1040	Pro	DEO	344	0	6	6	1	4	1	0	0
		NH_4^+	DEO	579	0	21	14	7	2	3	2	0
		Urea	DEO	1857	0	836	38	28	4	0	6	0
	SAA1032	Pro	DEO	269	0	31	11	4	5	2	0	0
		NH_4^+	DEO	341	0	84	16	6	4	0	6	0
		Urea	DEO	246	0	4	4	0	2	2	0	0

Table 3.4. Contiued

Source of sample	Strain[a]	N-source[b]	Treat[c]	*niaD* or *nirA*	*crn*	Total *cnx*	Genetically analysed *cnx*	ABC	E	F	G	H
This study	SAA1023a	Pro	DEO	660	0	15	8	3	1	2	1	1
		NH_4^+	DEO	125	0	0	0	0	0	0	0	0
		Urea	DEO	75	0	0	0	0	0	0	0	0
	$SAA1023_b$	Pro	DEO	280	0	318	35	14	9	9	4	2
		NH_4^+	DEO	212	0	230	38	8	11	9	5	2

(a) For full genotype of strains see Materials and Methods, section 2.1. **(b)** sole nitrogen source used, Pro denotes proline, glu denotes glutamate, U.A denotes uric acid, $NH4^+$ denotes ammonium, Arg, denotes arginine, Asn denotes asparagine. **(c)** denotes the treatment used, spont denotes spontaneous, NTG denotes N-methyl-N-Nitro-Nitrosoguanidine, DEO denotes 1,2,7,8-Diepoxyoctane.

Table 3.4. Continued.

Source of sample	Strain[a]	N-source[b]	Treat[c]	*niaD* or *nirA*	*crn*	Total *cnx*	Genetically analysed *cnx*	ABC	E	F	G	H
D. J. Cove (1976 a)	*biA1*	Arg	spont	194	0	3	3	2	0	1	0	0
		Urea	spont	173	2	22	22	10	4	4	3	1
		Glu	spont	194	0	6	6	6	0	0	0	0
		Asn	spont	148	22	24	24	15	1	6	1	1
		U.A	spont	178	6	14	14	8	1	3	1	1
	anA1 yA1	Arg	spont	182	0	17	17	2	2	9	4	0
		Urea	spont	172	5	20	20	14	0	3	0	3
		Glu	spont	192	0	7	7	6	1	0	0	0
		Asn	spont	158	13	26	26	18	0	3	4	1
		U.A	spont	159	12	25	25	10	4	4	2	5
	biA1 puA2 pyroA4	Arg	spont	204	1	1	1	1	0	0	0	0
		Urea	spont	196	1	8	8	5	0	3	0	0
		Glu	spont	208	0	0	0	0	0	4	0	0
		Asn	spont	184	2	14	14	8	0	3	0	0
		U.A	spont	199	3	4	4	1	0	0	2	0

Table 3.4 Continued.

Source of sample	Strain	N-source [b]	Treat [c]	*niaD* or *nirA*	*crn*	Total *cnx*	Genetically analysed *cnx*	ABC	E	F	G	H
D. J. Cove, (1976 a).	*yA1* *puA2*	Arg	NTG	162	42	144	144	73	30	20	10	11
	puA2	Arg	NTG	145	30	148	148	73	32	26	4	13
	yA1 *puA2*	Arg	spont	184	1	13	13	5	0	7	1	0
		U.A	spont	179	2	18	18	3	0	13	2	0
	puA2	Arg	spont	189	0	11	11	9	0	2	0	0
		U.A	spont	169	1	30	30	12	1	1	16	0
	yA1 *puA2*	Arg	NTG	96	1	48	48	39	6	3	0	0
		U.A	NTG	90	33	34	34	21	6	6	1	0
	puA2	Arg	NTG	102	0	72	72	44	4	17	4	3
		U.A	NTG	107	35	35	35	23	5	7	0	0

(b) denotes nitrogen source used, arg, denotes arginine, glu denotes glutamate, Asn denotes asparagine, U.A denotes uric acid. **(c)** denotes the treatment used, spont denotes spontaneous, NTG denotes N-methyl-N-Nitro-Nitrosoguanidine.

CHAPTER FOUR

CHARACTERISATION OF *crn* MUTANTS.

4.1 The Objectives Of This Research Section.

The existence of more than one nitrate transport system is an open question. The main aim of this particular part of the work is to explore the possibility of the existence of further nitrate transport encoding genes.

A second but minor aim of this section of work was to try to clarify the possibility of the existence of caesium sensitive only (independent of chlorate resistance), genes and determine if such genes are genetically linked to chlorate resistance.

That the presence of caesium prevents the growth of *crn* mutants on certain nitrogen sources (e.g. nitrate) a further aim was to try to find a growth selection system to clone new *crn* genes.

The fourth aim was to generate temperature-conditional *crn* mutants and to study these biochemically and moleculary. The question regarding the number of transport systems and the effect of mutational changes on the activities of this system(s) could be answered in this way.

4.2 Isolation Conditions Of *crn* Mutants Which Have Been Genetically Analysed.

The confirmation of chlorate resistant mutants with a *crn* phenotype were carried out according to the growth criteria listed in Table 3.1. Two sets of test plates (see section 3.3) were inoculated. One set was incubated at 25°C and the other at 37°C. Certain generated *crn* mutants showed complete chlorate sensitivity at the non-selection temperature whilst resistant at the selection temperature. Such mutants were taken and classified first as temperature-conditional mutants and second were classified further according to the growth criteria listed in Table 4.1. Twelve temperature-conditional *crn* mutants were generated from a total of 425 confirmed *crn* chlorate resistant mutants (approx 3% of the total chlorate mutants isolated). Nine out of these 12 *crn* mutants were thermo-sensitive i.e. resistance to chlorate at 37°C but sensitivity at 25°C. Three *crn* mutants showed a cryso-sensitive phenotype i.e. growth in the presence of chlorate at 25°C, but chlorate sensitivity at 37°C.

4.3 Genetic Recombination Analysis Of *crn* Mutants.

Forty seven out of the 425 *crn* strains (including the temperature-conditional mutants) were analysed genetically . The isolation conditions of these mutants are given in Table 4.2. One of the main aims of this line of research was to explore the possibility of unearthing further nitrate transport genes through mutant selection and subsequent meiotic recombination analysis between

newly isolated *crn* mutants during this work and the previously identified *crnA* mutants such as *crnA1*. Therefore, pair-wise sexual crosses were performed between *crnA1* mutant and each of the 47 new but confirmed phenotypically *crn* mutants.

Since *crn* mutants have an additional phenotype to that of chlorate resistance, namely sensitivity to caesium when grown on nitrate (see section 1.7.4), both phenotypes were followed in genetic crosses.

Surprisingly, genetic recombination analysis indicated that there were further putative nitrate transport gene(s) in addition to *crnA1*. In this regard, only 19 of the 47 genetically analysed mutants were found to map in the *crnA* locus i.e. no chlorate sensitive caesium resistant recombinants were observed in such pair-wise crosses with *crnA1* (Table 4.3). Amongst the 19 *crnA* mutants 4 were found to be phenotypically thermo-sensitive. The 28 of the 47 *crn* mutants analyed were found to locate in other loci, since recombinant progeny (i.e. non-parental types) *vis-a- vis* chlorate sensitivity were observed (Tables 4.4, 4.5, 4.6).

Pair-wise sexual crosses were set up between two (namely *crn993* ^{ts} and *crn1088* ^{cs}) alleles amongst the 28 non-*crnA* mutants. Genetic recombination analysis indicate that these two mutants mapped in the same locus as each other which segregates freely from the *crnA* locus. Both were designated *crnB* mutants. Further

attempts were carried out to identify the rest of the non-*crnA* mutants. Sexual pair-wise crosses were set up between *crnB993*,^{ts} *crnB1088* ^{cs} and the rest of the non-*crnA* mutants (i.e. 26 mutants). Genetic recombination analysis of such sexual crosses indicate that 23 of these 26 *crn* mutants locate in *crnB*, i.e. no chlorate sensitive, caesium resistant recombinants progeny were observed between *crnB993* ^{ts} and a further 21 *crnB* mutants (Table 4.7). Additionally, chlorate sensitive recombinants were not observed when the mutant *crnB1088* ^{cs} was crossed to the 10 mutants which had been already crossed to *crnB993*. This analysis supported the conclusion that these 10 mutants indeed lie in the *crnB* locus (Table 4.8). In summary, a total of 23 *crnB* mutants were identified. Within these 23 *crnB* mutants, 17 were found to be non-conditional, 3 thermo-sensitive and 3 cryso-sensitive. Moreover, the remaining 5 non-*crnA*, non-*crnB* mutants indicated that there was at least one further *crn* locus which is distinct from *crnA* and *crnB* loci (Tables 4.6, 4.9, and 4.10). One of these five mutants was designated as *crnC* (namely *crnC1056*) and sexual pair-wise crosses were set up between this *crnC* mutant and thus far unidentified 4 strains. Genetic recombination analysis of these crosses (Table 4.11) indicate that four out of the five mutants mapped in *crnC* locus, whilst one mutant identified in a fourth locus designated *crnD* locus (i.e.*crnD1116*).

To be certain of the existance of the *crnD* gene, pair-wise crosses were performed between this *crnD* mutant and other three mutants in *crnC* (excluding *crnC1056*). Table 4.6 shows that *crnD1116* does indeed map in a locus which is genetically different in location from *crnA*, *crnB*, or *crnC*. Regarding the *crnC* locus, two mutants were identified as temperature-sensitive, while the other two were temperature non-conditional.

To confirm these results further, genetic pair-wise crosses were set up in all genetic combinations, i.e. between *crnA1* mutant and a representative non-conditional *crn* mutant from each locus (i.e. *crnB967*, *crnC1056*, *crnD1116*). Additionally, pair-wise crosses were performed between *crnB* and *crnC*, *crnB* with *crnD* and *crnC* with *crnD* mutants. The results in Table 4.12 shows that there are indeed four different and distinct *crn* loci involved. A summary of all the genetically analysed *crn* mutant work is shown in Table 4.13.

Another interesting phenomena was noted during observation of the genetic analysis of *crn* mutants. That is wherever chlorate-sensitive and caesium-resistant recombinant progeny observed in pair-wise crosses between *crn* mutants mapped genetically in two different *crn* loci, new recombinant progeny were observed in low numbers. Such progeny showed resistance to both chlorate and caesium (i.e. chlorate resistant-caesium resistant, designated R R) others showed sensitivity to both chlorate and caesium (i.e. chlorate

sensistive-caesium sensitive, designated S S) (Tables 4.4, 4.5, 4.6 and 4.12).

Further outcrosses between wild-type and representatives of each *crn* locus (i.e. *crnA1*, *crnB993*,^{ts} *crnB1088*,^{cs} *crnB967*, *crnC1056* and *crnD1116*) were set up and analysed. Detailed results of such outcrosses between wild-type and *crnA1*, *crnB993* ^{ts} or *crnB1088* ^{cs} are shown in Table 4.14. Additionally, outcrosses were set up between wild-type and temperature non-conditional representative *crn* mutants from each *crn* locus and the results are shown in Table 4.15. The results obtained indicate that the new recombinant progeny that showed chlorate resistant-caesium resistant (designated R R) or chlorate sensistive-caesium sensitive (designated S S) occurred only in *crnA*, *crnB* or *crnC* recombinant progeny but, not in *crnD* progeny.

As a further attempt to confirm this point, further genetic crosses were carried out. First, chlorate sensitive-caesium sensitive progeny *crnB993*:7w, *crnB993*:17g, *crnB1088*:2w, *crnB1088*:10w and *crnB1088*:13g (i.e. which came from outcrosses between wild-type with either *crnB993* ^{ts} or *crnB1088* ^{cs}) were crossed to each other and to *crnA1* mutant, detailed results are shown in Table 4.16. These results indicate that when chlorate sensitive-caesium sensitive recombinant events crossed to each other, all progeny showed complete sensitivity to both chlorate and caesium (i.e. all progeny showed parental phenotypes). In contrast, when such chlorate

sensitive-caesium sensitive recombinant progeny were crossed to *crnA1* mutant, non-parental phenotypic progeny such as chlorate sensitive-caesium sensitive or chlorate resistant-caesium resistant occurred in low numbers.

Furthermore, representative chlorate sensitive-caesium sensitive recombinant progeny (i.e. 7w 993 and 13y C1056) that came about as a result of outcrossing either *crnB993* ^{ts} or *crnC1056* to the wild-type strain were crossed to each other. The results of such crosses indicate that these two chlorate sensitive-caesium sensitive progeny mapped in the same locus (i.e. all analysed progeny from these crosses were of parental phenotypes). Additionally, pair-wise crosses between chlorate resistant-caesium resistant mutants (i.e. 4y B967 and 1g C1056) that came about as a result of outcrossing either *crnB967* or *crnC1056* to the wild-type also indicate that such recombinant progeny map in the same locus (Data not shown) which is different from the chlorate sensitive-caesium sensitive locus (i.e. all analysed progeny showed highly resistance to both chlorate and caesium). These results may possibly suggest the presence of two further genes. First, a gene designated *chlA* which is chlorate resistance, and caesium resistant. Second, a gene designated *cesA* which is caesium sensitive and chlorate sensitive.

4.4 Growth Tests.

4.4.1 Growth On Nitrate.

All progeny that resulted from sexual crosses between *crn* mutants of different loci, showed wild-type levels of growth on different concentrations (i.e. from 0.5, 1 mM, 5 mM and 10 mM nitrate) of nitrate .

4.4.2 Heterokaryons Complementation.

Functional complementation in heterokaryons were attempted at 37°C between representative mutants of the four different *crn* loci, on glucose minimal medium, pH6.5 containing caesium chloride (50 mM) and sodium nitrate (10 mM). Complementation therefore done on the basis of the restoration of growth on caesium nitrate medium. The results shown in Table 4.17 indicate that *crn* mutants from the same locus or from a different *crn* locus, were unable to complement each other. It is unclear why this test was not successful, one of the possibilities is that both strains showed sensitivity to caesium (i.e. dominant trait) so mutants can not complement each other. Its success would have significantly reduced the effort of analysing the mutants by sexual crosses.

4.4.3 Growth Tests On Chlorate Or Caesium With Different Nitrogen Sources.

crnA Mutants.

Growth tests analysis on minimal media containing chlorate or caesium with different nitrogen sources was carried for all *crnA* mutants (Table 4.18) indicate that these mutants showed resistance to chlorate with proline, arginine or urea (except mutant *crnA* 1087 showed sensitivity with urea). In contrast, some of the mutants showed complete sensitivity to chlorate when glutamate was the sole nitrogen source. These mutants include thermo-sensitive strains *crn538* ^{ts} and *crn300* ^{ts} (Table 4.18).

On the other hand, certain *crnA* mutants showed different levels of resistance to caesium with different nitrogen sources. Data presented in Table 4.18 shows that approximately 55% to 65% of *crnA* mutants showed intermediate levels (Scored as R/S) of resistance to caesium when proline, arginine, or urea were used as the sole nitrogen source. However, only 15% showed this level of resistance with glutamate, nitrate, or nitrite. In contrast, 85% of *crnA* mutants showed complete sensitivity to caesium with nitrate, whilst the remaining 15% were sensitive to caesium with urea.

crnB Mutants.

All *crnB* mutants (with the exception of the three cryso-sensitive mutants, *crnB1088* ^{cs}, *crnB1077* ^{cs} and *crnB456* ^{cs}) were

highly resistant to chlorate with either proline, arginine, or urea as the sole nitrogen source (Table 4.19). However, all *crnB* mutants (except the non-conditional *crnB935*) were either completely sensitive or showed an intermediate level of resistance when glutamate was the sole nitrogen source with chlorate. Concerning caesium sensitivity, all *crnB* mutants were highly sensitive to caesium with nitrate as the sole nitrogen source, whilst varying levels of resistance were observed in these mutants with other nitrogen sources used (Table 4.19).

crnC Mutants.

All *crnC* mutants (with the exception of non-conditional *crnC1056*) showed high resistance to chlorate with proline, arginine, glutamate, or urea was used as the sole nitrogen source. In contrast, all *crnC* mutants were very sensitive to caesium with glutamate as the sole nitrogen source (Table 4.20). These results suggested that such complete sensitivity to caesium might be a useful selection marker for the future cloning of this gene.

crnD Mutant.

The *crnD1116* mutant showed high resistance to chlorate with proline, arginine or urea. Additionally, *crnD1116* strain showed complete sensitivity to chlorate with glutamate. However, *crnD1116* mutant show caesium sensitivity with arginine, nitrate, or nitrite (Table 4.20).

4.4.4 Growth Tests Of Chlorate Sensitive-Caesium Sensitive Strains.

Progeny which came as a result of the outcross between each of two *crnB* mutants (*crnB993* ^{ts} or *crnB1088* ^{cs}) to wild-type strain and showed complete sensitivity to both chlorate and caesium, were tested on nitrate, chlorate with proline and caesium with nitrate at both 25°C and 37°C. Additionally, such mutants were also tested on caesium with different nitrogen sources including nitrate, nitrite, ammonium, glutamate, or proline, where the mutants were incubated at the selection temperature of the parental strain (see legend of Table 4.21). All the chlorate sensitive-caesium sensitive mutants tested showed wild-type levels of growth on nitrate (10 mM) at both selection temperatures (i.e. 25°C and 37°C). Moreover, all mutants showed complete sensitivity to chlorate with all nitrogen sources used; however all isolates were resistant to caesium in the presence of either ammonium or proline.

4.5 Temperature-Conditional *crn* Mutants.

Twelve *crn* mutants were identified as temperature-conditional mutants (i.e. showed sensitivity to chlorate at the non-selection temperature). Following the growth criteria listed in Table 4.1, nine mutants were classified as thermo-sensitive *crn* mutants i.e. showing sensitivity to chlorate at 25°C, and wild-type levels of growth on nitrate at both temperatures. Three mutants were identified as cryso-sensitive *crn* mutants i.e. showing chlorate sensitivity at 37°C and wild-type levels of growth on nitrate at both

selection temperatures. The data in Figure 4.1 shows chlorate resistance patterns at three different temperatures (i.e. 25°C, 37°C, and 42°C) of a representative temperature-conditional *crn* mutant from each *crn* locus i.e. locus A (*crnA538*), locus B (*crnB993*) and locus C (*crnC640*). The results presented in Figure 4.2 shows the growth patterns of these representative mutants on nitrate as the sole nitrogen source at the same temperatures used with chlorate (i.e. 25°C, 37°C, and 42°C).

The results obtained from genetic recombination analysis show that these temperature-conditional *crn* mutants map in three different loci, namely *crnA*, *crnB* and *crnC*. Four *crn* mutants of the thermo-sensitive type map in locus A, three in locus B and two *crn* in locus C, whereas, all the three cryso-sensitive mutants fall into locus B.

A summary of the temperature-conditional *crn* mutants is shown in Table 4.22. All these mutants were tested for (1) chlorate resistance with proline as the sole nitrogen source (2) resistance to caesium with nitrate (3) resistance to caesium with nitrite, all tests at three different temperatures (i.e. 25°C, 30°C and 37°C). The results shown in Table 4.23 indicates that all temperature-sensitive *crn* mutants (from the different loci) showed a high level of resistance (Scored level 5) to chlorate at 37°C, but completely sensitive at other temperatures (Scored 0). In contrast, the three cryso-sensitive *crn* mutants (i.e. *crnB1088* ^{cs}, *crnB1077* ^{cs} and *crnB456* ^{cs}) were highly

Figure 4.1 Chlorate Resistance Patterns Of Representative Temperature-Conditional *crn* Mutants.

Representative mutants were tested at three different temperatures (i.e.25°C, 37°C, 42°C) on glucose minimal medium at pH 6.5 containing potassium chlorate (300 mM) and proline (10 mM) as the sole nitrogen source.

crnA538: representative temperature-sensitive *crn* mutant in locus A.
crnB993: representative temperature-sensitive *crn* mutant in locus B.
crnC640: representative temperature-sensitive *crn* mutant in locus C.
crnB1088 or *crnB1077*: representative cryso-sensitive (i.e. cold-sensitive) *crn* mutant in locus B.

Representative temperature-sensitive *crn* mutants were selected on chlorate at 37°C, show resistance to chlorate at high temperatures (i.e. 37°C, 42°C) and sensitivity at the low temperature (i.e. 25°C).

Representative cryso-sensitive *crn* mutants were selected on chlorate at 25°C, show resistance to chlorate at lower temperature (i.e. 25°C) and sensitivity at high temperatures (i.e. 37°C, 42°C).

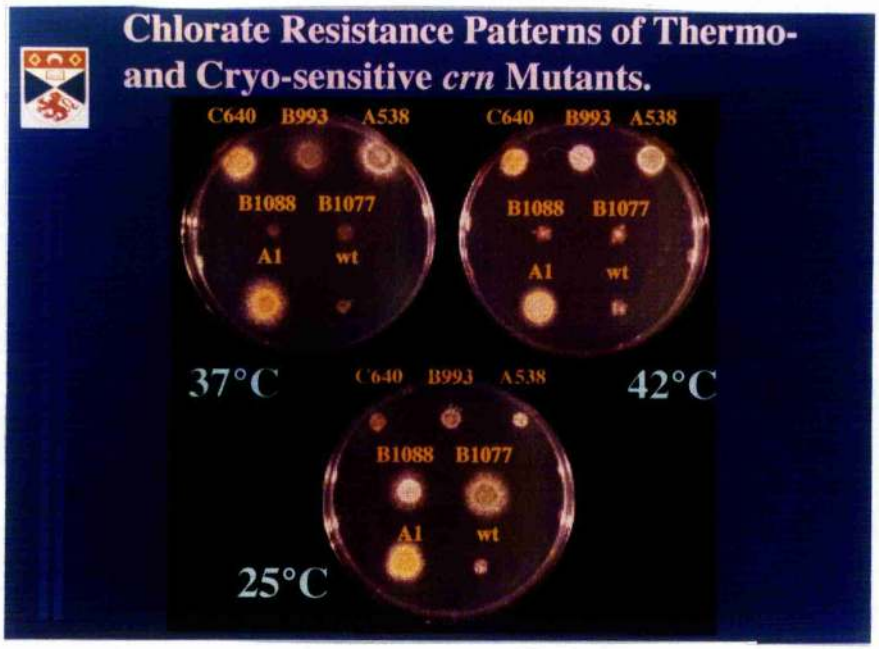

Chlorate Resistance Patterns of Thermo- and Cryo-sensitive *crn* Mutants.
C640 B993 A538
B1088 B1077
A1 wt
37°C
C640 B993 A538
B1088 B1077
A1 wt
42°C
C640 B993 A538
B1088 B1077
A1 wt
25°C

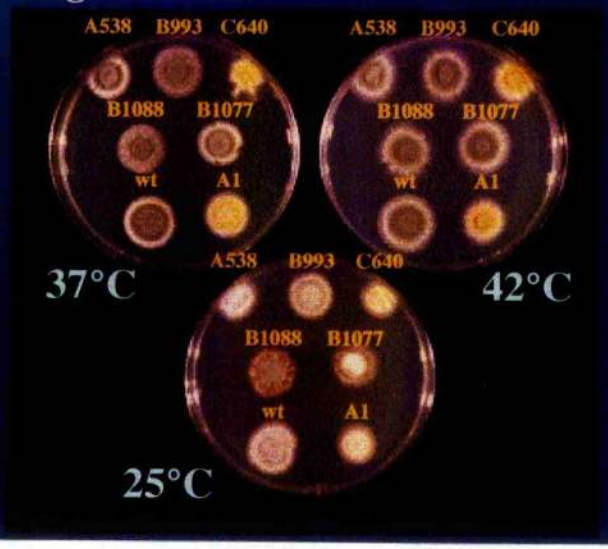
Figure 4.2 Growth Patterns Of Representative Temperature-Conditional *crn* Mutants On Nitrate As The Sole Nitrogen Source.

Representative mutants were tested at three different temperatures (i.e.25°C, 37°C, 42°C) on glucose minimal medium at pH 6.5 containing potassium chlorate (300 mM) and proline (10 mM) as the sole nitrogen source.

crnA538: representative temperature-sensitive *crn* mutant in locus A.
crnB993: representative temperature-sensitive *crn* mutant in locus B.
crnC640: representative temperature-sensitive *crn* mutant in locus C.
crnB1088 or *crnB1077*: representative cryso-sensitive (i.e. cold-sensitive) *crn* mutant in locus B.

All representative temperature-conditional crn mutants showed wild-type levels of growth on nitrate at the three different growth temperatures (25°C, 37°C, and 42°C).

Growth of *crn* Mutants on Nitrate as Sole Nitrogen source.



resistant at the lower temperatures (i.e. 25°C or 30°C) and completely sensitive at 37°C. Regarding caesium sensitivity phenotype, *crnA300* and *crnA496* showed intermediate levels of resistance to caesium with nitrate at either 37°C or 30°C but not at 25°C. In contrast, *crnA946* and *crnA496* showed resistance to caesium with nitrite at 37°C.

The *crnB* mutants, *crnB456*, *crnB1088* and *crnB974* exhibited level one to caesium with nitrate at 37°C and levels 3, 4 and 5 respectively to caesium resistance with nitrite. However, the two temperature-sensitive *crnC* mutants (*crnC635* and *crnC640*) showed level 3 resistance to caesium with nitrate and level 4 to caesium with nitrite at 37°C. Both *crnC* mutants had the same level of resistance (Scored level 2) to caesium with either nitrate or nitrite at 30°C (Table 4.23).

Further growth tests were carried out, where all temperature-conditional *crn* mutants were tested for resistance to caesium with different nitrogen sources at both 25°C and 37°C. The data obtained (Table 4.24) indicate that all temperature-conditional mutants were completely sensitive to caesium at 25°C with either nitrate or urea as the sole nitrogen source. In contrast, all mutants showed intermediate levels of caesium resistance (R/S) at 37°C with either proline or urea. The wild-type was completely resistant at both temperatures.

4.6 Nitrate Transport Activity.

Net nitrate uptake values were measured in wild-type and two representative mutants from each locus (except the crnD locus which is represented by only one mutant). Mycelial cells were grown for 8, 12, or 16 h in liquid minimal medium at 37°C with urea (5 mM) as the sole nitrogen source. The uptake system(s) were induced by nitrate (10 mM) 100 minutes before harvesting the cells. Net uptake values were obtained at 37°C after 10 or 20 min from the addition of nitrate (500 μM).

4.6.1 Net Nitrate Uptake Capacity In Various Temperature Non-Conditional *crn* Mutants.

Net Nitrate Uptake Values Obtained After 10 Minutes From The Addition Of Nitrate To The Assay Media.

In young cells (i.e. 8 h grown cells) net nitrate uptake values obtained at 37°C indicate that there was a 6-fold reduction in net uptake capacity in *crnA* strains as compared with wild-type under the same induced growth conditions (Table 4.25). Representative *crnB* (i.e. *crnB967* and *crnB976*) and *crnD1116* mutants showed approximately 4-fold reduction in net nitrate uptake capacity, whilst *crnC* mutants (i.e. *crnC1056* and *crnC561*) possessed 50% of the wild-type uptake rate.

At a medium age (i.e. after 12 h incubation) net nitrate uptake capacity was reduced 3-fold in *crnA1* and *crnD1116* mutants, whilst

there was a 7-fold reduction in the uptake capacity in the *crnA688* strain as compared to the wild-type under the same induced conditions. Mutants *crnB967*, *crnB976*, *crnC1056* and *crnC561* showed approximately 2 to 3 fold reduction compared to the wild-type.

In older mycelia (i.e. after 16 h incubation) there was no difference which could be detected between *crnA1* and the wild-type strain in net nitrate uptake capacity. There was little or no difference in this capacity in *crnA688* and *crnC1056* mutants. The net uptake capacity was reduced approximately 2 fold in both *crnB* mutants (i.e. *crnB967* and *crnB976*), *crnC561* and *crnD1116* as compared to the wild-type values.

Net Nitrate Uptake Capacity In *crn* Mutants After 20 Minutes Of Nitrate Addition To Assay Media.

In younger (i.e. 8 h incubation) cells representative mutants in *crnA* (i.e. *crnA1* and *crnA688*) and *crnB* (*crnB967* and *crnB976*) loci have shown approximately 6-fold reduction in net nitrate uptake capacity as compared to wild-type cells under the same induction conditions. Uptake capacity was reduced approximately 2-fold in *crnC1056* and *crnC561* and *crnD1116* mutants.

At the intermediate (i.e. 12 h incubation) stage there was a 4-fold reduction in the net nitrate uptake capacity in both *crnA* (i.e. *crnA1* and *crnA688*) and in *crnD1116* mutants. The uptake

was reduced by 2-fold in *crnB* 967, *crnB976* , *crnC1056* and *crnC561* mutants.

In older (i.e. 16 h incubation) cells there was no or little difference in the net nitrate uptake capacity between *crnA* mutants and wild-type strain under the same induction conditions. In contrast, uptake was reduced approximately 2-fold in *crnB967*, *crnB976*, *crnC1056*, *crnC561* and *crnD1116* mutants.

4.6.2 Net Nitrate Uptake Capacity In Various Thermo-Sensitive *crn* Mutants.

Net nitrate uptake rates were determined in wild-type, four thermo-sensitive *crnA* mutants (i.e. *crnA538*, *crnA300*, *crnA496* and *crnA946*), two thermo-sensitive mutants in *crnB* (i.e. *crnB974* and *crnB1030)* or *crnC* (i.e. *crnC635* and *crnC640*) loci. Mycelial cells were grown for 8 hours at 37°C with urea (5 mM) as the sole nitrogen source and induced for 100 min by nitrate (10 mM) before harvesting. Net nitrate uptake values were determined at either 25°C or 37°C after 10 or 20 minutes from the addition of nitrate (500 μM) to assay media. Additionally net uptake values were determined (as controls) in cells having no nitrate in the assay medium. The results are shown in Table 4.26.

Net Nitrate Uptake Values After 10 Minutes From Addition Of Nitrate To Assay Media.

Net uptake data, presented in Table 4.26, indicate that in

younger cells (8 h) there was approximately 50% reduction in net nitrate uptake capacity in all thermo-sensitive *crn* mutants assayed at 37°C as compared to the wild-type assayed where all strains are induced under the same conditions. However, at 25°C there was no or little difference in net uptake capacity between wild-type and all tested *crn* mutants, with the exception of strain *crnB1030* where there was a 50% reduction as compared to the wild-type strain.

Net Nitrate Uptake Values After 20 Minutes From Addition Of Nitrate To Assay Media.

At 25°C assay temperature the younger (i.e. 8 h incubation) cells of all representative thermo-sensitive *crn* mutants did not show significant difference in net nitrate uptake capacity as compared to wild-type under the same inducing conditions. At the 37°C assay temperature, the net uptake capacity was dropped to 50% in all *crn* mutants as compared to wild-type.

4.7 Nitrate Reductase Activity In Wild-Type And Various Non-Conditional *crn* Mutants.

Two representative non-conditional *crn* mutants from each locus (i.e. *crnA1*, *crnA688*, *crnB967*, *crnB976*, *crnC1056* and *crnC561*) were assayed with *crnD1116* and wild-type. Mycelial cells were grown on nitrate at 37°C for 8, 12, or 16 h and assayed for nitrate reductase activity at 25°C.

In younger cells (i.e 8 h incubation) and medium age (i.e.

12 h) cells, mutations in all *crn* genes have reduced the enzyme nitrate reductase activity approximately 50% as compared to wild-type levels under the same induction and assay conditions (Table 4.27). However, these mutations showed approximately wild-type level of enzyme activity in older cells (i.e. 16 h incubation). Enzyme levels in wild-type intermediate (i.e 12 h grown cells) cells had dropped approximately by one third compared to the level in 8 h cells and in 16 h old cells this wild-type enzyme level has dropped by 4-fold as compared to that in younger cells and approximately 2-fold as compared to wild-type level in medium age cells. Furthermore, mutations in all *crn* loci have lowered enzyme levels 50% in medium age as compared to younger cells (i.e. 8 h incubation). Additionally, these mutations showed 2-fold reduction in enzyme levels in older cells as compared to younger cells, whilst such older cells showed around 33% reduction in enzyme level as compared with cells of medium age.

4.8 Nucleotide Characterisation Of Designated *crnA* Mutants.

A number of *crnA* mutants were sequenced at the DNA level (Data not shown). The wild-type allele has previously been sequenced (Unkles *et. al.*, 1991) (see introduction). It must be noted that the entire protein coding section was analysed in these mutants, in this way. Changes could be clearly seen for *crnA1*, *crnA1009* and *crnA1087* mutants and these are highlighted in Table 4.28. Unexpectedly no changes were observed in mutants *crnA517*, *crnA521*, *crnA688*, *crnA760*, *crn775* or *crn1025* .

4.9 Discussion.

Genetic recombination analysis indicate that there are three novel nitrate transport genes designated *crnB*, *crnC* and *crnD* in addition to the previously identified *crnA1*. Twelve mutants were identified as temperature-conditional strains. Amongest these nine mutants were characterised as thermo-sensitive ones, whilst three were cryso-sensitives. Genetic recombination analysis indicate that the nine thermo-sensitive mutants map in three loci, four mutants in *crnA*, three in *crnB* and two in *crnC*. The three cryso-sensitive mutant alleles locate in *crn* B.

The progeny generated in all sexual crosses between *crn* mutants within the same locus or between mutants of different loci showed wild-type levels of growth on different nitrate concentrations. It may have been expected that double mutants would lead to a loss of growth on nitrate but that was not the case with these results.

Mutant complementation in heterokaryons between *crn* mutants (mutants within the same locus or between mutants of different loci) has failed for reasons unclear to me. Successful heterokaryon complementation would have significantly reduced the effort of analysing the mutants by sexual crosses

Genetically analysed mutants were tested on chlorate or caesium with different nitrogen sources. These growth tests indicate

that all *crn* mutants showed resistance to chlorate with either proline, arginine, or urea, whereas, all *crn* mutants showed sensitivity with glutamate except *crnC* mutants. All *crnB* and 85% of *crnA* mutants showed complete sensitivity to caesium with nitrate as the sole nitrogen source, whereas, all *crnC* mutants showed this sensitivity with glutamate. The *crnD1116* mutant showed this sensitivity with either arginine, nitrate, or nitrite. These results may suggest that this high hypersensitivety to caesium could be a successful selection marker for cloning the newly isolated putative transport genes.

Moreover, crosses between crn mutants can give rise to unusual progeny; sensitive to both chlorate and caesium or resistant to both chlorate and caesium.

Genetic recombination analysis of progeny from pair-wise outcrosses between wild-type and *crnA1* mutant allele showed that recombinant progeny of chlorate resistant-caesium resistant {R R} type can be recovered but no caesium sensitive-chlorate sensitive {S S} were revealed. Such new recombinants are unlike *crn* mutants which are chlorate resistant but caesium sensitive. These data may suggest that both chlorate resistance and caesium resistance co-segregate as one single gene or two tightly-linked genes in *crnA* mutant alleles i.e. chlorate and caesium resistance are part of *crnA* mutation. Basis of original crosses is that chlorate resistance and caesium sensitive phenotypes are manifested from the same gene defect.

When such pair-wise crosses were set up between *crnB* mutant alleles (i.e. *crnB993* or *crnB967*) and the wild-type strain (*biA1*) both the above mention new recombinants (i.e. R R and S S) as well as wild-type and *crnB* parental phenotypes were observed. Additionally, when further crosses were performed between *crnC* mutant allele (i.e. *crnC1056*) and the wild-type, these new R R and S S recombinants were revealed, as well as the parental phenotypes. These data may indicate the presence of two further genes. The first could be designated *chlA* and leads to chlorate resistance with caesium resistance (i.e. unlike *crn* mutants which are chlorate resistant but caesium sensitive). A second, designated *cesA* leads to caesium sensitive and chlorate sensitive (i.e. again unlike *crn* mutants). These two putative genes appear unlinked to *crn* genes or to each other.

Pair-wise crosses performed between *chlA* recombinants (i.e. showed chlorate resistance with caesium resistance) in *crnB* (i.e. GH 4y B967) and *crnC* (i.e. GH 1g C1056) revealed that all progeny are phenotypically parental progeny (R R type). These data may suggest that both *crnB* and *crnC* mutants either have the same *chlA* gene or there are two chlorate genes close to each other (i.e. tightly linked) which is unlikely.

Additionally, when chlorate sensitive-caesium sensitive recombinant progeny (i.e. designated *cesA* mutants) in *crnB* (i.e. GH 7w B993) and *crnC* (i.e. GH 13y C1056) mutant alleles were crossed to each other again all are phenotypically parental

progeny (i.e. S S). These findings may suggest that both *crnB* and *crnC* mutants either contain a further but identical caesium gene (i.e. *cesA*) or two different genes close to each other.

Genetic recombination analysis showed that when sexual pair-wise crosses were performed between *crnD1116* and any of the *crnA*, *crnB* or *crnC* mutant alleles chlorate sensitive recombinant progeny were revealed. These data would indicate that *crnD* is indeed a different *crn* gene from *crnA*, *crnB*, or *crnC* but no *chlA* or *cesA* recombinant progeny were observed. However, such data would indicate that both *crnA* and *crnD* genes have the same chlorate gene which is part of *crn* mutation since no R R or S S progeny revealed from the outcrosses. A summary of these conclusions is shown in Table 4.29.

Nitrate uptake data obtained after 20 minutes from the addition of nitrate to assay media indicate that the net nitrate uptake capacity in younger cells (i.e. 8 h incubation) was reduced approximately 6-fold in the non-conditional *crnA* or *crnB* mutants. The *crnC* and *crnD* mutants showed 2-fold reduction as compared to wild-type. At the intermediate (i.e. 12 h incubation) stage the net nitrate uptake capacity was reduced approximately 4-fold in either *crnA* or *crnD* mutants, whilst the uptake was reduced by 2-fold in *crnB* and *crnC* mutants. In contrast, older *crnA* mutant cells showed none or little difference in net nitrate uptake capacity as compared to wild-type, whilst the uptake capacity in *crnB*, *crnC*, or *crnD* mutants was reduced approximately 2-fold.

Net nitrate uptake data obtained from young (i.e. 8 h incubation) cells of thermo-sensitive mutants showed that there was a 50% reduction in net nitrate uptake in all *crn* mutants assayed at 37°C, whereas, little or no difference was observed at 25°C as compared to wild-type under the same induction conditions with the exception of strain *crnB1030* where there was a 50% reduction.

Nitrate reductase activities measured at different ages of representative *crn* mutants indicate that nitrate reductase levels were reduced approximately 50% in either young or medium aged cells. Whereas, enzyme levels were the same in older cells as compared to wild-type levels. All combined net nitrate uptake and nitrate reductase activity data indicate that there was approximately a 50% reduction in either net nitrate uptake or enzyme levels in all new *crn* mutants of young or medium aged cells. The defect in these mutants is unclear, since certain of these *crn* mutants have reduced the uptake capacity of nitrate in young cells but not in older ones. These results may suggest that these *crn* genes may specify specific components for a complex nitrate transport system, where a defect in some components or part of the complex system would make the system active at certain age of cells but not at other ages. Alternatively, *crn* genes may represent distinct nitrate transport systems independent of each other, where certain system becomes functional in young cells while others in older cells. Another possibility is that *crnA* is a distinct nitrate transporter (Brownlee and Arst, 1983; Unkles *et. al.*, 1991) whilst the other *crn*

products are working as molecular chaperones through the passage of CRNA protein from ribosomes to the cell membrane. Any defect in such chaperones would lead to passage of defective CRNA protein and that may lead to represent a nitrate transport system which is active at certain age (i.e. older cells) of cells but not in younger cells.

There is a parallel relationship between net nitrate uptake values and nitrate reductase levels where there was a reduction in both levels in younger cells compared to no or little difference in older cells. This may suggest that nitrate transport requires functional nitrate reductase activity in order to be active which would agree with previously obtained results i.e. as NR activity increases with age nitrate uptake increases (Brownlee and Arst, 1983).

Nucleotide characterisation of *crnA* mutants showed that, the *crnA1009* mutation leads to a frame shift that affects the protein in the second last membrane spanning domain and thereafter (i.e. near the terminal), which may suggest that such a mutation does not affect nitrate transport as much as *crnA1087* mutation which is a single amino acid change, glycine to glutamate in the seventh membrane spanning domain (i.e. the protein in this domain is altered by one amino acid and that would suggest such a mutation is very important for nitrate transport. Unexpectedly no change was noticed in 6 more *crnA* mutants i.e. *crnA517*, *crnA521*, *crnA688*, *crnA760*, *crnA775* and *crnA1025*. These mutations could be either in the upstream

promoter region or in closely linked genes. It is unclear from sequence analysis whether the status of these mutations should be designated *crnA*. Genetic recombination results do suggest that these mutations map to the *crnA* locus. Therefore, such mutants are now designated *crnA**.

Table 4.1. Criteria Used For The Classification Of Thermo- And Cryso-Sensitive Mutants.

Mutant	Selection Temperature	Resistant To Chlorate 37°C	Resistant To Chlorate 25°C	Nitrate 37°C	Nitrate 25°C	Adenine 37°C	Adenine 25°C
Cnx ^{ts}*	37°C	R	R	-	+	-	-
Cnx ^{ts}	37°C	R	R	-	+	-	+
Cnx ^{cs}	25°C	R	R	+	-	+	-
Crn ^{ts}	37°C	R	S	+	+	+	+
Crn ^{cs}	25°C	S	R	+	+	+	+
biA1 cnxH4 *		R	R	-	-	-	-
biA1 *		S	S	+	+	+	+

Mutants were tested on glucose supplmented minimal medium (with vitamins), pH 6.5 containing sodium nitrate (5 mM), or adenine (5 mM) as sole nitrogen source, or potassium chlorate (300 mM) and nitrogen source (10 mM) depending on the nitrogen source used at selection. R: denotes resistant, S: denotes sensitive, symbol (-): denotes no growth, symbol (+): denotes wild-type level of growth. symbol (*): denotes temperature sensitive *cnx* mutant on nitrate, but showed the behaviour of non-conditional mutant (ie. totally mutant) on hypoxanthine at both temperatures.

Table 4.2. Summary Of Isolation Conditions For *crn* Mutants Which Have Been Genetically Analysed.

a
Two wild-type strains *biA1* (G1) and *yA2 pyroA4* (A220) were used in the treatment.
b
The wild-type *yA2 pyroA4* strain was used in this treatment.
c
The wild-type *biA1* strain was used in this treatment.
*
Chlorate resistant *crn* mutants were tested at both temperatures (25° C and 37° C) at least three times on glucose supplemented minimal medium, pH 6.5 with :

(1) Potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source.

(2) Adenine (5 mM) as the sole nitrogen source.

(3) Sodium nitrate (5 mM) as the sole nitrogen source. Mutants were classified according to the criteria listed in Table 3.1.

- Growth analyses were carried at both temperatures (25° C and 37° C).

Treatment	Spontaneous				N-methyl-N-nitro-nitrosoguanidine (NTG)					1,2,7,8-Diepoxyoctane (DEO)	
Mutagenised strain	Wild-type[a]		Wild-type[b]		Wild-type[a]					Wild-type[c]	
N-source	Proline		Uric acid	Glutamate	Proline		Glutamate	Uric acid		Glutamate	
Selection temperature	25° C	37° C	37° C	37° C	25° C	37° C	37° C	25° C	37° C	25° C	37° C
crn mutant	*crnA749*	*crnA688*	*crnC635*	*crnA496*	*crnA116*	*crnA517*	*crnA216*	*crnA1087*	*crnA946*	*crnB1075*	*crnB1030*
	crnA756		*crnC640*		*crnB443*	*crnA521*	*crnA300*	*crnB1088*	*crnA1009*	*crnB1077*	
	crnA760				*crnB456*	*crnA538*	*crnD1116*		*crnA1025*		
	crnA770					*crnB523*			*crnA1034*		
	crnA771					*crnB546*			*crnB935*		
	crnA775					*crnB552*			*crnB939*		
	crnB700					*crnC561*			*crnB967*		
	crnB703								*crnB969*		
	crnB706								*crnB972*		
									crnB974		
									crnB976		
									crnB993		
									crnB1023		
									crnB1061		
									crnC1056		

a
Two wild-type strains were used in this treatment, *biA1* (G1) and *yA2 pyroA4* (A220).

b
The wild-type strain *yA2 pyroA4* was used in this treatment.

c
The wild-type strain *biA1* was used in this treatment.

Table 4.3. Genetic Crosses Between *crnA1* Mutant And All *crnA* Mutants.

a

The mutant number designated, is the original number that was given to the mutant when it was first selected and isolated on chlorate·

b

For each cross, one perithecium was analysed (except with *crnA538,* where two perithecia were analysed), 23 green progeny and 23 yellow progeny from each cross were analysed (except *crnA538,* in which 43 progeny were analysed from each colour).

c

The Progeny were tested on glucose minimal medium (plus supplements) at pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source. Also progeny were tested on the same medium but with caesium chloride (50 mM) and sodium nitrate (10 mM) as the sole nitrogen source. In the analysis, both parents and the wild-type were included in each test as controls.

d

(R): denotes highly resistant, (S): denotes completely sensitive (no growth): Total number of progeny which showed resistance to chlorate and caesium.

e

Total number of progeny which showed sensitivity to chlorate and resistance to caesium.

f

Total number of progeny which showed resistance to both chlorate and caesium.

g

Total number of progeny which showed sensitivity to both chlorate and caesium.

h

Nitrate non-utilisers: None, chlorate sensitive recombinants: None, caesium resistant recombinants None.

- All phenotypic analysis were carried out at 37°C.

crnA1 [a] Crossed to the following mutants	Total [b] progeny screened	[c,d] ClO_3^- Cs^+	[e] ClO_3^- Cs^+	[f] ClO_3^- Cs^+	[g] ClO_3^- Cs^+
		R S	S R	R R	S S
crnA1009	46	46	0	0	0
crnA1025	46	46	0	0	0
crnA749	46	46	0	0	0
crnA116	46	46	0	0	0
crnA775	46	46	0	0	0
crnA688	46	46	0	0	0
crnA760	46	46	0	0	0
crnA216	46	46	0	0	0
crnA756	46	46	0	0	0
crnA1087	46	46	0	0	0
crnA521	46	46	0	0	0
crnA517	46	46	0	0	0
crnA771	46	46	0	0	0
crnA770	46	46	0	0	0
crnA1034	46	46	0	0	0
crnA300^{ts}	46	46	0	0	0
crnA496^{ts}	46	46	0	0	0
crnA946^{ts}	46	46	0	0	0
crnA538^{ts}	86	86	0	0	0

Table 4.4 Genetic Crosses Between *crnA1* Mutant And All *crnB* Mutants.

a

The mutant number designated is the original number that was given to the mutant when it was first selected and isolated on chlorate·

b

For each cross, one perithecium was analysed, 20 or 23 green progeny and 20 or 23 yellow progeny from each cross were analysed.

c

The Progeny were tested on glucose minimal medium (plus supplements) at pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source. Also progeny were tested on the same medium but with caesium chloride (50 mM) and sodium nitrate (10 mM) as the sole nitrogen source. In the analysis of progeny, both parents and the wild-type were included in each test as controls.

d

(R): denotes highly resistant, (S): denotes completely Sensitive (no growth):
Total number of progeny which showed resistance to chlorate and caesium.

e

Total number of progeny which showed sensitivity to chlorate and resistance to caesium.

f

Total number of progeny which showed resistance to both chlorate and caesium.

g

Total number of progeny which showed sensitivity to both chlorate and caesium.
-Nitrate Non-utilisers: None. All phenotypic analysis were done at 37°C.

crnA1 [a] crossed to the following mutants	Total [b] progeny	ClO_3^- [c] [d] Cs^+		ClO_3^- [e] Cs^+		ClO_3^- [f] Cs^+		ClO_3^- [g] Cs^+		ClO_3^- sensitive recombinants		Cs^+ resistant recombinants	
		R	S	S	R	R	R	S	S	No	%	No	%
crnB976	52	38		12		2		0		12	23%	14	27%
crnB935	46	26		14		1		5		19	41%	15	33%
crnB546	46	38		6		1		1		7	15%	8	18%
crnB523	46	28		3		10		5		8	17%	18	39%
crnB552	46	33		7		5		1		8	17%	13	28%
crnB443	46	38		8		0		0		8	17%	8	18%
crnB967	40	23		9		8		0		9	23%	17	43%
crnB1061	52	39		10		1		2		12	23%	13	25%
crnB706	46	36		10		0		0		10	22%	10	22%
crnB1075	46	35		8		3		0		8	17%	11	24%
crnB939	46	29		12		5		0		12	26%	17	37%

Table 4.4. Continued..

crnA1 [a] crossed to the following mutants	Total [b] progeny	ClO_3^- [c d] Cs^+		ClO_3^- [e] Cs^+		ClO_3^- [f] Cs^+		ClO_3^- [g] Cs^+		ClO_3^- sensitive recombinants		Cs^+ resistant recombinants	
		R	S	S	R	R	R	S	S	No	%	No	%
crnB972	46	23		17		4		2		19	41%	23	50%
crnB700	46	35		7		2		2		9	20%	11	24%
crnB703	46	27		13		6		0		13	28%	19	41%
crnB1023	52	44		8		0		0		8	15%	8	15%
crnB969	46	33		9		4		0		9	20%	13	28%
crnB456^{cs}	46	24		18		0		2		20	43%	22	48%
crnB1088^{cs}	40	23		10		7		0		10	25%	17	43%
crnB1077^{cs}	40	25		8		7		0		8	20%	15	38%
crnB993^{ts}	40	30		5		5		0		5	13%	10	25%
crnB974^{ts}	40	30		10		3		0		10	25%	10	25%
crnB1030^{ts}	40	26		9		5		0		9	23%	14	35%

Table 4.5. Genetic Crosses Between *crnA1* Mutant And All *crnC* Mutants.

a

The mutant number designated, is the original number that was given to the mutant when it was first selected and isolated on chlorate·

b

For each cross one perithecium was analysed, 20 or 23 green progeny and 20 or 23 yellow progeny were analysed.

c

The Progeny were tested on glucose minimal medium (plus supplements), at pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source. Also progeny were tested on the same medium but with caesium chloride (50 mM) and sodium nitrate (10 mM) as the sole nitrogen source. In the analysis of progeny, both parents and the wild-type were included in each test as controls.

d

(R): denotes highly resistant, (S): denotes completely Sensitive (no growth):
Total number of progeny which showed resistance to chlorate and caesium.

e

Total number of progeny which showed sensitivity to chlorate and resistance to caesium.

f

Total number of progeny which showed resistance to both chlorate and caesium.

g

Total number of progeny which showed sensitivity to both chlorate and caesium.

-Nitrate Non-utilisers: None. All phenotypic analysis were done at 37°C.

crnA1 [a] crossed to the following mutants	Total progeny [b]	ClO_3^- [c] [d]	Cs^+	ClO_3^- [e]	Cs^+	ClO_3^- [f]	Cs^+	ClO_3^- [g]	Cs^+	ClO_3^- Sensitive recombinants		Cs^+ resistant recombinants	
		R	S	S	R	R	R	S	S	No	%	No	%
crnC561	46	36		9		1		0		9	20%	10	22%
crnC1056	46	34		6		5		1		7	15%	11	24%
crnC640^{ts}	46	31		11		3		1		12	26%	14	30%
crnC635^{ts}	46	23		12		11		0		12	26%	23	50%

Table 4.6. Genetic Crosses Between *crnD1116* Mutant And All *crnA*, *crnB* And *crnC* Mutants.

a

The mutant number designated, is the original number that was given to the mutant when it was first selected and isolated on chlorate·

b

For each cross one perithecium was analysed, 23 progeny from each colour(green, yellow) were analysed .

c

The Progeny were tested on glucose minimal medium (plus supplements), at pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source. Also progeny were tested on the same medium but with caesium chloride (50 mM) and sodium nitrate (10 mM) as the sole nitrogen source. In the analysis of progeny, both parents and the wild-type were included in each test as controls.

d

(R): denotes Resistant, (S): denotes sensitive (no growth): Total number of progeny which showed resistance to chlorate and sensitivity caesium.

e

Total number of progeny which showed sensitivity to chlorate and resistance to caesium.

f

Total number of progeny which showed resistance to both chlorate and caesium.

g

Total number of progeny which showed sensitivity to both chlorate and caesium.

-Nitrate Non-utilisers: None. All phenotypic analysis were done at 37°C.

crnD1116 [a] crossed to the following mutants	Total progeny [b]	ClO_3^- Cs+ [c d]		ClO_3^- Cs+ [e]		ClO_3^- Cs+ [f]		ClO_3^- Cs+ [g]		ClO_3^- sensitive recombinants		Cs+ resistant recombinants	
		R	S	S	R	R	R	S	S	No	%	No	%
crnA1	52	32		17		3		0		17	33%	20	38%
crnB1088^{cs}	46	26		10		0		1		10	22%	10	22%
crnB993^{ts}	46	32		8		2		4		12	26%	14	30%
crnC561	46	25		15		6		0		15	32%	21	46%
crnC640^{ts}	46	20		16		10		0		16	35%	26	57%
crnC635^{ts}	46	16		17		13		0		17	37%	30	65%

Table 4.7. Genetic Pair Wise Crosses Between *crnB*993^{ts} Mutant And Various *crnB* Mutants.

a

The mutant number designated, is the original number that was given to the mutant when it was first selected and isolated on chlorate·

b

For each cross one perithecium was analysed (except*crnB969* two perithecia were analysed), 23 progeny from each color(green, yellow or white) were analysed (except *crnB969* ,43 progeny were analysed from each color).

c

The Progeny were tested on supplemented glucose minimal medium (plus supplements), at pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source. Also progeny were tested on the same medium but, with caesium chloride (50 mM) and sodium nitrate (10 mM) as the sole nitrogen source. In the analysis of progeny, both parents and the wild-type were included in each test as controls.

d

(R): denotes highly resistant, (S): denotes complete Sensitivity (no growth): Total number of progeny which showed resistance to chlorate and caesium.

e

Total number of progeny which showed sensitivity to chlorate and resistance to caesium.

f

Total number of progeny which showed resistance to both chlorate and caesium.

g

Total number of progeny which showed sensitivity to both chlorate and caesium.

h

Nitrate non-utilisers: None, chlorate sensitive recombinants: None, caesium resistant recombinants: None

-All phenotypic analysis were done at 37°C.

crnB993 [a] Crossed to the following mutants	Total [b] progeny	[c,d] ClO_3^-	Cs^+	[e] ClO_3^-	Cs^+	[f] ClO_3^-	Cs^+	[g] ClO_3^-	Cs^+
		R	S	S	R	R	R	S	S
crnB939	46	46		0		0		0	
crnB972	46	46		0		0		0	
crnB976	46	46		0		0		0	
crnB1023	46	46		0		0		0	
crnB935	46	46		0		0		0	
crnB1061	46	46		0		0		0	
crnB552	46	46		0		0		0	
crnB703	46	46		0		0		0	
crnB706	69	69		0		0		0	
crnB 546	46	46		0		0		0	

Table 4.7. Continued.

crnB993 [a] Crossed to the following mutants	Total progeny [b]	[c,d] ClO_3^-		Cs^+ [e]		ClO_3^- [f]	Cs^+	ClO_3^- [g]	Cs^+
		R	S	S	R	R	R	S	S
crnB523	46	46		0		0		0	
crnB443	46	46		0		0		0	
crnB700	46	46		0		0		0	
crnB1075	46	46		0		0		0	
crnB967	46	46		0		0		0	
crnB969	92	92		0		0		0	
crnB456^{cs}	46	46		0		0		0	
crnB1077^{cs}	46	46		0		0		0	
crnB1094	46	46		0		0		0	
crnB974^{ts}	46	46		0		0		0	
crnB1030^{ts}	46	46		0		0		0	

Table 4.8. Genetic Crosses Between *crnB1088* ^{cs} Mutant And Various *crnB* Mutants.

a

The mutant number designated, is the original number that was given to the mutant when it was first selected and isolated on chlorate·

b

For each cross, one perithecium was analysed (except with *crnB1030* where two perithecia were analysed), 23 progeny from each color(green, yellow or white) were analysed from each cross,(except with *crnB1030* where 46 progeny were analysed from each color).

c

The Progeny were tested on glucose minimal medium (plus supplements), at pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source. Also progeny were tested on the same medium but, with caesium chloride(50 mM) and sodium nitrate (10 mM) as the sole nitrogen source. In the analysis of progeny, both parents and the wild-type were included in each test as controls.

d

(R): denotes highly resistant, (S): denotes completely Sensitive (no growth): Total number of progeny which showed resistance to chlorate and caesium.

e

Total number of progeny which showed sensitivity to chlorate and resistance to caesium.

f

Total number of progeny which showed resistance to both chlorate and caesium.

g

Total number of progeny which showed sensitivity to both chlorate and caesium.

h

Nitrate non-utilisers: None, chlorate sensitive recombinants: None, caesium resistant recombinants: None

-All phenotypic analysis were done at 37°C, or 25° C for the cryso-sensitive.

crnB1088 [a] Crossed to the following mutants	Total [b] progeny	ClO_3^- [c,d] Cs^+		ClO_3^- [e] Cs^+		ClO_3^- [f] Cs^+		ClO_3^- [g] Cs^+	
		R	S	S	R	R	R	S	S
crnB976	46	46		0		0		0	
crnB1023	46	46		0		0		0	
crnB935	46	46		0		0		0	
crnB552	46	46		0		0		0	
crnB546	46	46		0		0		0	
crnB523	46	46		0		0		0	
crnB443	46	46		0		0		0	
crnB967	46	46		0		0		0	
crnB1077 [cs]	46	46		0		0		0	
crnB993 [ts]	46	46		0		0		0	
crnB974 [ts]	46	46		0		0		0	
crnB1030 [ts]	92	92		0		0		0	

Table 4.9. Genetic Crosses Between *crnB993* Mutant And *crnC* Mutants.

a

The mutant number designated, is the original number that was given to the mutant when it was first selected and isolated on chlorate.

b

For each cross, one perithecium was analysed, 23 progeny from each colour (green, yellow or white) were analysed.

c

The Progeny were tested on glucose minimal medium (plus supplements), at pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source. Also progeny were tested on the same medium with caesium chloride (50 mM) and sodium nitrate (10 mM) as the sole nitrogen source. In the analysis of progeny both parents and the wild-type were included in each test as controls.

d

(R): denotes resistant, (S): denotes completly sensitive (no growth): Total number of progeny which showed resistance to chlorate and sensitivity caesium.

e

Total number of progeny which showed sensitivity to chlorate and resistance to caesium.

f

Total number of progeny which showed resistance to both chlorate and caesium.

g

Total number of progeny which showed sensitivity to both chlorate and caesium.

-Nitrate Non-utilisers: None. All phenotypic analysis were done at 37°C.

crnB993 [a] crossed to the following mutants	Total[b] progeny	ClO_3^- [c] [d] Cs^+		ClO_3^- [e] Cs^+		ClO_3^- [f] Cs^+		ClO_3^- [g] Cs^+		ClO_3^- sensitive recombinants		Cs^+ resistant recombinants	
		R	S	S	R	R	R	S	S	NO	%	NO	%
crnC561	69	46		23		0		0		23	33%	23	33%
crnC640 [ts]	69	53		16		0		0		16	23%	16	23%
crnC635 [ts]	69	51		18		0		0		18	26%	18	26%

Table 4.10. Genetic Crosses Between *crnB1088* Mutant And *crnC* Mutants.

a

The mutant number designated, is the original number that was given to the mutant when it was first selected and isolated on chlorate.·

b

For each cross one perithecium was analysed, 23 progeny from each colour(green, yellow or white) were analysed.

c

The Progeny were tested on glucose minimal medium (plus supplements), at pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source. Also progeny were tested on the same medium with caesium chloride (50 mM) and sodium nitrate (10 mM) as sole nitrogen source. In the analysis of progeny both parents and the wild-type were included in each test as controls.

d

(R): denotes resistant, (S): denotes completly sensitive (no growth: Total number of progeny which showed resistance to chlorate and sensitivity caesium.

e

Total number of progeny which showed sensitivity to chlorate and resistance to caesium.

f

Total number of progeny which showed resistance to both chlorate and caesium.

g

Total number of progeny which showed sensitivity to both chlorate and caesium.

- Nitrate Non-utilisers: None. All phenotypic analysis were done at 37°C.

crnB1088[a] crossed to the following mutants	Total[b] progeny	[c] [d] ClO_3^-	Cs^+	[e] ClO_3^-	Cs^+	[f] ClO_3^-	Cs^+	[g] ClO_3^-	Cs^+	ClO_3^- sensitive recombinants		Cs^+ resistant recombinants	
		R	S	S	R	R	R	S	S	NO	%	NO	%
crnC561	69	54		15		0		0		15	22%	15	22%
crnC1056	46	33		13		0		0		13	28%	13	28%
crnC640[ts]	69	49		20		0		0		20	29%	20	29%
crnC635[ts]	69	50		19		0		0		19	28%	19	28%

Table 4.11. Genetic Crosses Between *crnC1056* Mutant And All *crnC* Mutants.

a

The mutant number designated, is the original number that was given to the mutant when it was first selected and isolated on chlorate·

b

For each cross, one perithecium was analysed, 23 progeny from each color(green, yellow) were analysed .

c

The Progeny were tested on glucose minimal medium (plus supplements), at pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source. Also progeny were tested on the same medium with caesium chloride (50 mM) and sodium nitrate (10 mM) as the sole nitrogen source. In the analysis of progeny, both parents and the wild-type were included in each test as controls.

d

Letter (R): denotes resistant, letter (S): denotes sensitive (no growth): Total number of progeny which showed resistance to chlorate and caesium.

e

Total number of progeny which showed sensitivity to chlorate and resistance to caesium.

f

Total number of progeny which showed resistance to both chlorate and caesium.

g

Total number of progeny which showed sensitivity to both chlorate and caesium.

h

Nitrate Non-utilisers: None, chlorate sensitive recombinants: None, caesium resistant recombinants: None

-All phenotypic analysis were done at 37°C.

crnC1056 [a] crossed to the following mutants	[b] Total progeny	[c,d] ClO_3^- R	[c,d] Cs^+ S	[e] ClO_3^- S	[e] Cs^+ R	[f] ClO_3^- R	[f] Cs^+ R	[g] ClO_3^- S	[g] Cs^+ S
crnC561	46	46		0		0		0	
crnC640 [ts]	46	46		0		0		0	
crnC635 [ts]	46	46		0		0		0	

Table 4.12. Genetic Pair Wise Crosses Between *crn* mutants map at 4 different loci, *crnA1*, *crnB967*, *crnC1056* and *crnD1116*.

a

Mutant number designated, is the original number that was given to the mutant when it was first selected and isolated on chlorate·

b.

Progeny were tested on glucose minimal medium (plus supplements), at pH 6.5, with

1- Ptassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source .

2- Caesium chloride (50 mM) and sodium nitrate (10 mM) as the sole nitrogen source.

3- Caesium chloride (50 mM) and sodium nitrite (10 mM) as the sole nitrogen source.

NB: In the analysis of progeny, both parents and the wild-type were included in each test as controls .

* (R): denotes highly resistant, (S): denotes completely sensitive (no growth).

***1**

Total number of progeny which showed resistance to chlorate and sensitivity to either caesium with nitrate or caesium with nitrite.

***2**

Total number of progeny which showed sensitivity to chlorate and resistance to either caesium with nitrate or caesium with nitrite.

***3**

Total number of progeny which showed sensitivity to caesium with nitrate, and resistance to either chlorate or caesium with nitrite.

***4**

Total number of progeny which showed resistance to all three media (chlorate, caesium with nitrate, and caesium with nitrite).

***5**

Total number of progeny which showed sensitivity to all three media (chlorate, caesium with nitrate, and caesium with nitrite).

* Nitrate non-utilisers: None, All phenotypic analysis were carried out at 37°C.

crn mutant [a]	Total progeny [b]	ClO_3^- pro	Cs^+ NO_3^-	Cs^+ NO_2^-	ClO_3^- pro	Cs^+ NO_3^-	Cs^+ NO_2^-	ClO_3^- pro	Cs^+ NO_3^-	Cs^+ NO_2^-	ClO_3^- pro	Cs^+ NO_3^-	Cs^+ NO_2^-	ClO_3^- pro	Cs^+ NO_3^-	Cs^+ NO_2^-
		R	S	S	S	R	R	R	S	R	R	R	R	S	S	S
crnA1 crossed to *crnB967* w	69	26			19			1			23			0		
crnA1 crossed to *crnB967* g	46	24			9			10			1			2		
crnA1 crossed to *crnC1056*	46	20			8			11			6			1		
crnA1 crossed to *crnD1116* a	46	20			9			14			2			1		
crnA1 crossed to *crnD1116* b	46	18			13			12			3			0		
crnA1 crossed to *crnB967* w	69	29			18			0			22			0		
crnB967 w crossed to *crnC1056*	69	34			15			0			18			2		
crnB967 w crossed to *crnD1116*	46	28			12			4			2			0		
crnC1056 crossed to *crnD1116*	46	23			8			0			14			1		

Table 4.13. Summary Of The Genetically Analysed *crn* Mutants.[a]

Locus / Mutant type	*crnA*	*crnB*	*crnC*	*crnD*
Non-conditional	15	16	2	1
Thermo-sensitive	4	3	2	0
Cryso-sensitive	0	3	0	0

[a] Total number of chlorate resistant mutants isolated 11807; *crn* mutants identified 425; thermo-sensitive mutants 9; cryso-sensitive mutants 3; genetically analysed mutants 46.

Table 4.14. Genetic Pair Wise Crosses Between Wild-Type And *crnA1*, *crnB993*, *crnB1088* Mutants.

a

Mutant numberdesignated, is the original number that was given to the mutant when it was first selected and isolated on chlorate·

b

For each cross, one perithecium was analysed, 23 progeny from each colour(green or yellow) were analysed .

c

Progeny were tested on glucose minimal medium (plus supplements), at pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as the sole nitrogen source. Also progeny were tested on the same medium but with caesium chloride (50 mM) and sodium nitrate (10 mM) as the sole nitrogen source. In the analysis of progeny both parents and the wild-type were included in each test as controls.

*: (R): denotes highly resistant, (S): denotes completely sensitive (no growth)

***1**

Total number of progeny which showed resistance to chlorate and sensitivity caesium.

***2**

Total number of progeny which showed sensitivity to chlorate and resistance to caesium.

***3**

Total number of progeny which showed resistance to both chlorate and caesium.

***4**

Total number of progeny which showed sensitivity to both chlorate and caesium.

- Nitrate Non-utilisers: None. All phenotypic analysis were carried out at 37°C.

crn mutant [a]	Total progeny [b]	ClO_3^- Cs^+ [c]		ClO_3^- Cs^+		ClO_3^- Cs^+		ClO_3^- Cs^+		NO_3^- none utilisers		ClO_3^- sensitive recombinants		Cs^+ resistant recombinants	
		R	S *1	S	R *2	R	R *3	S	S *4	No	%	No	%	No	%
crnA1	125	66		42		17		0		0	0%	42	34%	59	47%
crnB993^{ts}	46	20		13		6		7		0	0%	20	44%	19	41%
crnB1088^{cs}	46	24		17		0		5		0	0%	22	48%	17	37%

Table 4.15, Genetic Pair Wise Crosses Between Wild-Type And *crnA1*, *crnB967*, *crnC1056* and *crnD1116* Mutants .

a

Mutant numberdesignated, is the original number that was given to the mutant when it was first selected and isolated on chlorate·

b

For each sexual cross, more than one perithecium was analysed, in *crnA1* 6 perathecia, *crnB967* 5 *crnC1056* 2, and in *crnD1116* 2 perathecia were analysed. In each sexual cross half of the total progeny were analysed from each colour.

c

Progeny were tested on glucose minimal medium (plus supplements), at pH 6.5, with

1- Ptassium chlorate (300 mM) and proline (10 mM) as the sole nitrogen source .

2- Caesium chloride (50 mM) and sodium nitrate (10 mM) as the sole nitrogen source.

3- Caesium chloride (50 mM) and sodium nitrite (10 mM) as the sole nitrogen source.

NB: With *crnA1* progeny, in addition to 50 mM caesium chloride, 60 and 100 mM were used, and the results were the same as with 50 mM. In each cross both parents were included in each test as controls .

* (R): denotes highly resistant, (S): denotes completely sensitive (no growth).

***1**

Total number of progeny which showed resistance to chlorate and sensitivity to either caesium with nitrate or caesium with nitrite as the sole nitrogen source.

***2**

Total number of progeny which showed sensitivity to chlorate and resistance to either caesium with nitrate or caesium with nitrite as the sole nitrogen source.

*3
Total number of progeny which showed sensitivity to caesium with nitrate, and resistance to either chlorate or caesium with nitrite.
*4
Total number of progeny which showed resistance to all three media (chlorate, caesium with nitrate, and caesium with nitrite).
*5
Total number of progeny which showed sensitivity to all three media (chlorate, caesium with nitrate, and caesium with nitrite).
* Nitrate non-utilisers: None, Analysis was carried out at 37°C.

Table 4.15.

crn mutant [a]	Total progeny [b]	ClO_3^- pro	Cs^+ NO_3^-	Cs^+ NO_2^- [c]	ClO_3^- pro	Cs^+ NO_3^-	Cs^+ NO_2^-	ClO_3^- pro	Cs^+ NO_3^-	Cs^+ NO_2^-	ClO_3^- pro	Cs^+ NO_3^-	Cs^+ NO_2^-
		R	S	S*1	S	R	R*2	R	S	R*3	R	R	R*4	S	S	S*5
crnA1	385	178			148			59			0			0		
crnB967	576	225			288			0			63			0		
crnC1056	192	87			86			0			15			4		
crnD1116	287	73			214			0			0			0		

Table 4.16. Genetic Crosses Between Recombinant*crnB* Progeny Sensitive To Both Caesium And Chlorate And Between Sensitive Progeny And *crnA1*.

a

The white and green recombinant progeny 7w and 17g (w: denotes white colour, and g: denotes green colour, respectivily) are representitive mutant, that came as a result of outcrossing *crnB993* ^{ts} to wild-type strain (*biA1*). These mutant showed sensitivity to both chlorate (150 mM) with proline (10 mM) as sole nitrogen source, and caesium (50 mM) with nitrate (10 mM) as sole nitrogen source.

b

The white and green recombinant progeny 2w, 10w, and 13g (w: denotes white colour, and g: denotes green colour) are representitive mutant, that came as a result of outcrossing *crnB1088* ^{cs} to wild-type strain (*biA1*). These mutant showed sensitivity to both chlorate (150 mM) with proline (10 mM) as sole nitrogen source, and caesium (50 mM) with nitrate (10 mM) as sole nitrogen source.

c

Progeny were tested on supplemented glucose minimal medium plus supplements, pH 6.5, with potassium chlorate (150 mM) and proline (10 mM) as sole nitrogen source. in addition progeny were tested on the same medium with caesium chloride (50 mM) and nitrate (10 mM) as sole nitrogen source. In each cross both parents and the wild-type were included in each test as controls.

d

letter R: denotes highly resistant, letter S: denotes completely sensitive, and column R-S: denotes total number of progeny which showed resistance to chlorate and sensitivity to caeium.

e

Column S-R: denotes total number of progeny which showed sensitivity to chlorate and resistance to caesium.

f

Column R-R: denotes total number of progeny which showed resistance to both chlorate and caesium.

g

Column S-S: denotes total number of progeny which showed sensitivity to both chlorate and caesium.

***a**

In each outcross one perithecium was analysed, 23 progeny from each colour (green, yellow or white) were analysed .

***b**

For caesium designation recombinant progeny *crnB993* :7w, *crnB993*:17g were designated *cesA1* and *crnB1088* :13g, *crnB1088* :10w, and *crnB1088* :2w were designated *cesA2*.

***c**

Analysis was carried out at 37°C and 25° C in which similar results were obtained.

Table 4.16

sensitive progeny of *crn* mutants crossed to each other and to *crn A1*	Total progeny[c]	ClO_3^-[d] Cs^+		ClO_3^-[e] Cs^+		ClO_3^-[f] Cs^+		ClO_3^-[g] Cs^+		NO_3^- non utilisers		ClO_3^- sensitive recombinants		Cs^+ resistant recombinants	
		R	S	S	R	R	R	S	S	No	%	No	%	No	%
crnB993 :7w[a] and *crnB1088* :13g[b]	46	0		0		0		46		0	0%	46	100%	0	0%
crnB993 :17g[a] and *crnB1088* :10w[b]	46	0		0		0		46		0	0%	46	100%	0	0%
crnA1 and *crnB993* :7w[a]	68	43		5		4		16		17	25%	21	31%	9	13%
crnA1 and *crnB993* :7w[a]	69	45		7		0		17		14	20%	24	35%	7	10%
crnA1 and *crnB993* :7w[a]	69	43		8		3		15		10	15%	23	33%	11	16%
crnA1 and *crnB1088* :2w[b]	68	33		16		7		12		25	36%	29	42%	23	34%

Table 4.17. Genetic Analysis Of Representative *crn* Mutants By Functional Complementation In Heterokaryon.

Heterokaryon complementation test between the following *crn* mutants.																	
crnA / *crnA*		*crnA* /*crnB*		*crnA* /*crnC*		*crnA* / *crnD*		*crnB* / *crnB*		*crnB* / *crnC*		*crnB* / *crnD*		*crnC* / *crnC*		*crnC* / *crnD*	
A1	775	A1	939	A1	1056	A1	1116	967	939	976	1056	993	1116	1056	635	1056	1116
A1	1009	A1	976	A1	635	1009	1116	967	976	976	635	976	1116	1056	640	640	1116
A1	688	A1	993	A1	640	688	1116	967	974	976	640	974	1116	1056	561	635	1116
A1	538	A1	1077	A1	561	775	1116	967	443	976	561	1023	1116	640	635	561	1116
A1	300	A1	967	1009	640			967	1023	967	1056	1030	1116	640	561		
1009	775	1009	972	688	1056			967	1030	967	635	1077	1116				
688	775	1009	976	688	635			967	993	967	640	967	1116				
300	775	1009	1077	688	640			967	1088	967	561						
300	538	688	967	688	561			967	1077	993	635						
688	538	688	1030	775	1056			967	972	993	640						
1034	1089	775	939					972	939	1030	640						
								972	1023	1023	1056						
								972	443	974	1056						
								974	1030	972	1056						
										1077	635						

a
Representative mutants, each number represent the original designated number that was given to the mutant when it was first selected and isolated on chlorate.

b
No complementation occurred between any of the mutants in each group. The was repeated at least three times.

c
Mutants were tested on supplemented glucose minimal medium, pH 6.5 with caesium chloride (50 mM) and sodium nitrate (10 mM) as nitrogen source, at 37° C.

Table 4.18 . Analysis Of *crnA* Mutants On Chlorate Or Caesium With Different Nitrogen Sources.

Strain[a]	b ClO_3^-				c Cs^+					
	pro	arg	glu	urea	pro	arg	glu	urea	NO_3^-	NO_2^-
crnA1009	R	R	S*	R	R	S	R	S	S	R/S
crnA775	R	R	R	R	R/S	R/S	R/S	R/S	S	R
crnA688	R	R	R	R	R/S	R/S	R	R/S	S	R
crnA1087	R	R	S	S	R/S	S	S	S	S	R/S
crnA1034	R	R	R	R	R/S	R/S	S	S	S	R
crnA760	R	R	R	R	R	R	S	R/S	S	R
crnA756	R	R	R	R	R/S	R/S	S	R/S	S	R
crnA521	R	R	R	R	R	R/S	R	S	S	R
crnA1025	R	R	R	R	R/S	R/S	S	S	S	R
crnA771	R	R	R	R	R/S	R/S	S	R/S	S	R
crnA770	R	R	S*	R	R/S	R/S	R	R/S	S	S
crnA216	R	R	S*	R	R/S	R/S	R	R/S	S	S
crnA116	R	R	S	R	R	R/S	R	R/S	S	S
crnA517	R	R	R	R	R	R/S	R	S	S	R
crnA749	R	S	S	R	R	S	R	S	R/S*	R
crnA496^{ts}	R	R	R	R	R	R/S	R	S	R/S*	R
crnA946^{ts}	R	R	S	R	R/S	S	S	S	S	R/S
crnA538^{ts}	R	R	S*	R	R/S	S	R/S	R/S	S	S
crnA300^{ts}	R	R	S*	R	R/S	R/S	R/S	R/S	R/S*	S
crnA1	R	R	S*	R	R/S	S	S	R/S	S	R/S
wild-type	S	S	S	S	R	R	R	R	R	R
niaD115	R	R	R	R	S	S	S	R/S	S	S
niaD1781	R	R	R	R	R	R/S	S	S	S	R/S

a
Mutant number is the original number that was given to the strain when it was selected on chlorate.

b
Mutants were grown in supplemented glucose minimal medium plus vitamins and potassium chlorate (150 mM), with proline (10 mM), arginine (10 mM), glutamate (10 mM) and urea (5 mM), at 37° C.

c
Mutants were grown in supplemented glucose minimal medium plus vitamins and cesium chloride (50 mM), with proline (10 mM), arginine (10 mM), glutamate (10 mM) and urea (5 mM), sodium nitrate (10 mM) and sodium nitrite (10 mM), at 37° C.

-letter (R): denotes highly resistant, letter (R/S): denotes intermediate, letter (S): denotes sensitive (no growth). growth tests have been done once (each mutant 5 replica).*Tests have been done twice.

Table 4.19. Analysis Of *crnB* Mutants On Chlorate Or Cesium With Different Nitrogen Sources.

a
Mutant number designated is the original number that was given to the strain when it was first selected and isolated on chlorate.

b
Mutants were grown in glucose minimal medium plus vitamins, with potassium chloride (150 mM) and proline (10 mM), arginine (10 mM), glutamate (10 mM), or urea (5 mM) as the sole nitrogen sources, at 37°C.

c
Mutants were grown in glucose minimal medium plus vitamins, with caesium chloride (50 mM) and proline (10 mM), arginine (10 mM), glutamate (10 mM) urea (5 mM), Nitrate (10 mM) or nitrite (10 mM) as the sole nitrogen sources, at 37°C.

- (R): denotes highly resistant, (R/S): denotes intermediate, (S): completly sensitive (no growth).

-growth tests have been carried out once.

*Tests have been carried out twice.

Strain[a]	b ClO_3^-				c Cs^+					
	pro	arg	glu	urea	pro	arg	glu	urea	NO_3^-	NO_2^-
crnB939	R	R	R/S*	R	R	R/S	R	R/S	S	R
crnB972	R	R	R/S*	R	R/S	R	S	S	S	R
crnB976	R	R	R/S*	R	R	R	R	R/S	S	R
crnB1023	R	R	S	R	R	R/S	R/S	R	S	R
crnB935	R	R	R*	R	R	R	S	R	S	R
crnB1061	R	R	S	R	R	S	R	R	S	S
crnB552	R	R	S	R	R	R/S	S	R/S	S	S
crnB703	R	R	S	R	R	S	S	R/S	S	R
crnB706	R	S	S	R	R	S	R	R	S	S
crnB546	R	R	S	R	R	R	R/S	R	S	S
crnB523	R	R	R/S	R	R	R	S	R	S	R
crnB443	R	R/S	S	R	R	R	R/S	R	S	S

Table 4.19. Continued.

Strain[a]	b ClO_3^-				c Cs^+					
	pro	arg	glu	urea	pro	arg	glu	urea	NO_3^-	NO_2^-
crnB700	R	R	R/S	R	R	R/S	R/S	R	S	R
crnB1075	R	R	R/S	R	R	S	S	R	S	R
crnB967	R	R	S*	R	R/S	S	R/S	S	S	S
crnB969	R	R	S	R	R/S	S	R/S	S	S	S
crnB456 cs	S*	S*	S*	S*	R/S	R/S	R/S	R/S	S*	R/S
crnB1077 cs	S	S	S	S	R/S	R	R/S	R/S	S	S
crnB1088 cs	S	S	S	S	R/S	R	R/S	R/S	S	R
crnB993 ts	R	S	S	R	S	S	R/S	S	S	S
crnB974 ts	R	R	S	R	R/S	R	S	R/S	S*	R
crnB1030 ts	R	R/S	S	R	R/S	S	S	R/S	S	S
crnA1	R	R	S*	R	R/S	S	R	R/S	S	R/S
wild-type	S	S	S	S	R	R	R	R	R	R
niaD115	R*	R*	R	R	S	S	S	S	S	S
niaD1781	R*	R*	R	R	R	R/S	S	S	S	R/S

Table 4.20. Analysis Of *crnC* And *crnD* Mutants On Chlorate Or Cesium With Different Nitrogen Sources.

Strain[a]	b ClO_3^-				c Cs^+					
	pro	arg	glu	urea	pro	arg	glu	urea	NO_3^-	NO_2^-
crnC1056	R	R*	S*	R	R	S	S	S	S	S
crnC561	R	R	R	R	R	R	S	R	S	R
crnC640 *ts*	R	R	R	R	R/S	R/S	S	R/S	R/S	R
crnC635 ts	R	R	R	R	R/S	S	S	R/S	R/S	R
crnD1116	R	R	S*	R	R/S	S	R/S	R/S	S	S
crnA1	R	R	S	R	R/S	S	R	R/S	S	R/S
wild-type	S	S	S	S	R	R/S	R	R/S	R	R
niaD115	R	R	R	R	S	S	S	S	S	S
niaD1781	R	R	R	R	R	R/S	S	S	S	R/S

a
Mutant number designated is the original number that was given to the strain when it was first selected and isolated on chlorate.

b
Mutants were grown in glucose minimal medium (plus vitamins), with potassium chloride (150 mM) and proline (10 mM), arginine (10 mM), glutamate (10 mM), or urea (5 mM) as sole nitrogen source, at 37° C.

c
Mutants were grown in glucose minimal medium (plus vitamins), with caesium chloride (50 mM) and proline (10 mM), arginine (10 mM), glutamate (10 mM), urea (5 mM) as sole nitrogen source, nitrate (10 mM) or nitrite (10 mM), at 37° C.

-(R): denotes highly resistant, (R/S): denotes intermediate, (S): denotes completly sensitive (no growth).
- Growth tests have been done once.
*Tests have been done twice.

Table 4.21 . Genetic Analysis Of Sensitive *crnB* Progeny To Both Chlorate And Caesium.

a

Serial number of the recombinant progeny which came as a result of out crossing *crnB1088* ^{cs} or *crnB993* ^{ts} to the wild-type, showed sensitivity to both chlorate and caesium.

b

Progeny were tested at both temperatures (25° C and 37° C) on supplemented glucose minimal medium with supplements, pH 6.5, and sodium nitrate (10 mM) as sole nitrogen source, potassium chlorate (150 mM) with proline (10 mM) as sole nitrogen source, and caesium chloride (50 mM) and sodium nitrate (10 mM) as sole nitrogen source.

c

Progeny were tested at 37° C for *crnB993* ^{ts} or 25° C for *crnB1088* ^{cs} on supplemented glucose minimal medium with vitamins, pH 6.5, and caesium chloride (50 mM) with sodium nitrate (10 mM) as sole nitrogen source, sodium nitrite (10 mM), glutamate (10 mM) or proline (10 mM) as nitrogen source. Both parents and the wild-type were included as controls.

Strain	[b] NO_3^-	NO_3^-	ClO_3^- Pro	ClO_3^- Pro	Cs^+ NO_3^-	Cs^+ NO_3^-	[c] Cs^+ NO_3^-	[c] Cs^+ NO_2^-	[c] Cs^+ NH_4^+	[c] Cs^+ glu	[c] Cs^+ pro
	37° C	25° C	37° C	25° C	37° C	25° C					
crnB1088 /13g[a]	+	+	S	S	S	S	S	S	R	S	R
crnB1088 /21g	+	+	S	S	S	S	S	S	R	S	R
crnB1088 /2w	+	+	S	S	S	S	S	S	R	S	R
crnB1088 /10w	+	+	S	S	S	S	S	S	R	S	R
crnB1088 /17w	+	+	S	S	S	S	S	S	R	S	R
crnB993 /119	+	+	S	S	S	S	S	S	R	S	R
crnB993 /13g	+	+	S	S	S	S	S	S	R	S	R
crnB993 /14g	+	+	S	S	S	S	S	S	R	S	R
crnB993 /17g	+	+	S	S	S	S	S	S	R	S	R
crnB993 /18g	+	+	S	S	S	S	S	S	R	S	R
crnB993 /7w	+	+	S	S	S	S	S	S	R	S	R
crnB993 /19w	+	+	S	S	S	S	S	S	R	S	R
crnB993 *ts*	+	+	R	S	S	S	S	S	R	R	S
crnB1088 cs	+	+	S	R	S	S	S	S	R	R	R
wild-type	+	+	S	S	R	R	R	R	R	S	R
crnA1	+	+	R	R	S	S	S	R/S	R	R	R

Table 22. Summary Of Temperature Conditional *crn* Mutants.

Mutant number	Locus[b]	Thermo-[c] sensitive	Cryso- [c] sensitive	Mutant designation	Other marker(s)	Strain number
300	A	√		*crnA300*^{ts}	*biA1*	GK.21
496	A	√		*crnA496*^{ts}	*yA2, pyroA4*	GK.22
538	A	√		*crnA538*^{ts}	*biA1*	GK.23
946	A	√		*crnA946*^{ts}	*biA1*	GK.24
456	B		√	*crnB456*^{cs}	*biA1*	GK.25
1077	B		√	*crnB1077*^{cs}	*biA1*	GK.26
1088	B		√	*crnB1088*^{cs}	*biA1*	GK.27
974	B	√		*crnB974*^{ts}	*biA1*	GK.28
993	B	√		*crnB993*^{ts}	*biA1*	GK.29
1030	B	√		*crnB1030*^{ts}	*biA1*	GK.30
635	C	√		*crnC635*^{ts}	*yA2, pyroA4*	GK.32
640	C	√		*crnC640*^{ts}	*yA2, pyroA4*	GK.33

a

Mutant number is the original number that was given to the mutant when it was selected on chlorate·

b

Locus A: as a result of no recombinants after crossing *crnA1* mutant with the designated *crnA* mutans. Locus B: as a result of recombinants after crossing *crnA1* mutant with the designated *crnB* mutants. Locus C: as a result of recombinants after crossing the designated *crnC* mutants to *crnA1* and *crnB* mutants.

c

Thermo-sensitive: showed resistance to chlorate at higher temperatures (37° C and 42° C) and sensitivity at lower temperatures (25° C and 30° C) . While the cryso-sensitive mutants showed the visa-versa.

*

mutants were tested on supplemented glucose minimal medium with vitamins, pH 6.5 and sodium nitrate (10 mM)as sole nitrpgen source, potassium chlorate (150 mM) with proline (10 mM) as sole nitrogen source.

Table 4.23. Growth Tests Of Temperature-Conditional *crn* Mutants On Chlorate Or Caesium With Different Nitrogen Sources.

a
The mutants were tested at temperatures indicated on glucose minimal medium (with supplements), pH 6.5, with potassium chlorate (200 mM) and proline (10 mM) as sole nitrogen source.
b
The Mutants were tested at temperatures indicated on glucose minimal medium (with supplements) pH 6.5, with caesium chloride (50 mM) and sodium nitrate (10 mM) as sole nitrogen source.
c
The Mutants were tested at temperatures indicated on glucose minimal medium (with supplements) pH 6.5, with caesium chloride (50 mM) and sodium nitrite (10 mM) as the sole nitrogen source.

- Figure (5): denotes highly resistant, figure (0): denotes completely sensitive (no growth).
- Growth tests have been done once.

Mutant designation	Resistance to								
	ClO_3^- + Pro [a]			$Cs^+ + NO_3^-$ [b]			$Cs^+ + NO_2^-$ [c]		
	37°C	30°C	25°C	37°C	30°C	25°C	37°C	30°C	25°C
Wild-type	0	0	0	5	5	1	5	3	1
crnA1	5	5	5	0	0	0	3	0	0
crnA300 ts	5	0	0	3	2	0	0	0	0
crnA538 ts	5	0	0	0	0	0	0	0	0
crnA946 ts	5	0	0	0	0	0	3	2	1
crnA496 ts	5	3	0	3	3	1	5	1	0
crnB456 cs	0	4	4	1	0	0	3	0	0
crnB1077 cs	0	4	4	1	0	0	0	0	0
crnB1088 cs	0	4	4	1	0	0	4	0	0
crnB974 ts	5	0	0	1	2	0	5	1	2
crnB993 ts	5	0	0	0	1	0	0	0	0
crnB1030 ts	5	0	0	1	3	1	0	0	0
crnB635 ts	5	0	0	3	2	1	4	2	0
crnC640 ts	5	0	0	3	2	1	4	2	0

Table 4.24. Analysis Of Representative Thermo- and Cryso-Sensitive Mutants In *crn* Loci On Caesium, With Different Nitrogen Sources.

Mutant[a]	$Cs^+ + NO_3^-$		Cs^+ + pro		Cs^+ + urea		Cs^+ + glu	
	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C
crnA538 ts	S	S	R/S	R/S	S	R/S	S	R/S
crnA300 ts	S	R/S	R/S	R/S	S	R/S	S	R/S
crnB1030 ts	S	S	R/S	R/S	S	R/S	S	S
crnB974 ts	S	S	R/S	R/S	S	R/S	S	S
crnB1088 cs	S	S	R	R/S	S	R/S	R	R/S
crnB1077cs	S	S	R	R/S	S	R/S	R	R/S
crnB456 cs	S	S	R	R/S	S	R/S	R	R/S
crnC635 ts	S	R/S	R/S	R/S	S	R/S	S	S
crnC640 ts	S	R/S	R/S	R/S	S	R/S	S	S
crnD1116	S	S	R/S	R/S	S	R/S	S	R/S
crnA1	S	S	R/S	R/S	S	R/S	S	S
niaD115	S	S	R/S	R/S	S	R/S	S	S
niaD1256	S	S	R/S	R/S	S	R/S	S	S
wild-type	R	R	R	R	R	R	R	R

a
Mutants were grown in glucose minimal medium with vitamins pH 6.5, and caesium chloride (50 mM), with sodium nitrate (10 mM), proline (10 mM), urea (5 mM) and glutamate (10 mM), at both temperatures (25°C and 37°C) as indicated.
- (R): denotes highly resistant, (R/S): denotes intermediate, (S): denotes completely sensitive(no growth).
- Growth tests have been done once.

Table 4.25. Net Nitrate Uptake In Wild-Type And Various Non-Conditional *crn* Mutant Cells.

[a]
Mycelia were grown at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source.
[b]
Sodium nitrate (10 mM) was added 100 min before harvesting the cells.
[c]
Net uptake values were expressed as nanomoles of nitrate removed from the medium per min per mg mycelia (blotted wet weight), and are the means ± standard deviation (SD) of three independent grow up experiments.
[d]
Representitive mutants at each *crn* locus, *crnA1* was described previously by Brownlee and Arst (1983).
[e]
Allele number designated is the original number that was given to the mutant when it was first selected and isolated on chlorate.
[f]
Nitrate uptake assays were carried out at 37°C, pH 6.5.
[*]
Nitrate concentration in the assay medium was 500 μM.

Age of mycelia at harvesting (h)[a]	Conc of nitrate (μM)[b]	Net nitrate uptake values after 10 min of incubation at 37°C.[c,d,e,f]							
		wild-type	*crnA1*	*crnA688*	*crnB976*	*crnB967*	*crnC1056*	*crnC561*	*crnD1116*
8	0	2.60±1.0	1.10 ±.74	0.74± 0.4	0.80 ±0.2	0.41±0.20	1.40 ±0.12	1.30 ±0.40	0.50 ±0.50
	500	30.84±8.6	3.90 ±1.3	5.30 ±1.1	7.10 ±1.3	8.30 ±0.40	13.90 ±0.8	13.20 ±2.0	8.50 ±0.50
12	0	2.70 ±1.8	3.00 ±0.8	0.20 ±0.1	0.70 ±0.13	0.74±0.40	1.30 ±0.23	1.40 ±0.30	0.44±0.13
	500	24.40 ±5.3	6.50 ±3.5	2.80 ±0.34	15.30 ±3.7	14.70 ±4.7	12.50 ±0.73	13.50 ±0.4	5.00 ±0.70
16	0	2.50 ±0.7	2.50 ± 1.2	1.80 ±0.7	0.50 ±0.13	0.70 ±0.13	1.10 ±0.40	1.50 ±0.23	1.65±0.13
	500	26.00±7.4	26.00±1.7	19.50 ±2.5	13.70 ±1.9	13.70 ±1.5	21.00 ±0.5	13.50 ±1.0	10.20 ±3.50
Age of mycelia at harvesting (h)[a]	Conc of nitrate[b] (mM)	Net nitrate uptake values after 20 min of incubation at 37°C.[c, d,e,f]							
		wild-type	*crnA1*	*crnA688*	*crnB976*	*crnB967*	*crnC1056*	*crnC561*	*crnD1116*
8	0	1.33±0.5	0.30 ±0.3	0.20 ±0.11	0.40 ±0.2	0.08±0.01	0.63±0.20	0.30 ±0.2	0.30 ±0.10
	500	11.55 ±1.3	2.20 ±0.8	1.90 ±0.13	2.30 ±0.8	2.70 ±0.2	6.40 ±0.30	6.30 ±0.3	4.60 ±0.30
12	0	0.70 ±0.4	0.50 ±0.3	0.20 ±0.11	0.20 ±0.06	0.60 ±0.4	0.50 ±0.30	0.30 ±0.0	0.10 ±0.06
	500	9.70 ±0.7	2.40 ±0.5	2.20 ±0.4	6.30 ±0.8	5.90 ±1.1	5.50 ±0.20	5.60 ±0.2	2.30 ±0.20
16	0	0.86±0.4	0.90 ±0.2	0.36±0.2	0.18±0.0	0.14±0.13	0.40 ±0.14	0.40 ±0.1	0.30 ±0.07
	500	12.00 ±1.2	12.30 ±0.8	11.30 ±0.5	5.70 ±0.84	6.00 ±0.9	5.90 ±0.60	5.60 ±0.3	5.30 ±0.60

Table 4.26. Net Nitrate Uptake In Wild-Type And Various Thermo-Sensitive *crn* Mutant Cells.

[a] Sodium nitrate (10 mM) was added 100 min before harvesting.

[b] Mycelia were grown for 8 h in glucose minimal medium supplemented with urea (5 mM) as sole nitrogen source, either at 37°C or at 25°C as indicated.

[c] Allele number designated is the original number that was given to the mutant when it was first selected and isolated on chlorate.

[d] Net uptake values were expressed as nanomoles of nitrate removed from the medium per min per mg mycelia (blotted wet weight), and are the means ± standard deviation (SD) of three independent grow up experiments.

[e] Representitive mutants at each *crn* locus.

[f] Nitrate uptake assays were carried out at 37°C, pH 6.5.

[*] Nitrate concentration in the assay medium was 500 μM.

Conc of[a] nitrate (μM)	Mycelia[b] growth Temp(°C)	Net nitrate uptake values after 10 min of incubation at 37°C.[c,d,e,f]								
		wild-type	*crnA538*	*crnA300*	*crnA496*	*crnA946*	*crnB974*	*crnB1030*	*crnC635*	*crnC640*
0	25	0.82 ± 0.37	0.62 ± 0.12	0.86 ± 0.11	0.77 ± 0.14	0.48 ± 0.04	0.33 ± 0.13	0.59 ± 0.07	0.60 ± 0.12	0.57 ± 0.11
	37	2.30 ± 0.53	0.56 ± 0.10	0.43 ± 0.09	0.59 ± 0.13	0.51 ± 0.33	0.43 ± 0.08	0.46 ± 0.22	0.73 ± 0.10	0.83 ± 0.15
500	25	15.14 ± 2.40	12.18 ± 3.50	10.15 ± 0.50	13.49 ± 1.40	12.20 ± 0.90	12.89 ± 0.64	9.50 ± 2.60	13.79 ± 2.20	14.72 ± 2.80
	37	27.58 ± 5.30	11.42 ± 1.00	15.56 ± 0.70	13.10 ± 1.10	12.74 ± 1.30	14.33 ± 0.41	14.10 ± 0.31	15.00 ± 0.30	15.10 ± 0.80
Conc of[a] nitrate (μM)	Mycelia[b] growth Temp(° C)	Net nitrate uptake values after 20 min of incubation at 37°C.[c,d,e,f]								
		wild-type	*crnA538*	*crnA300*	*crnA496*	*crnA946*	*crnB974*	*crnB1030*	*crnC635*	*crnC640*
0	25	0.53 ± 0.17	0.53 ± 0.10	0.58 ± 0.09	0.39 ± 0.07	0.33 ± 0.15	0.30 ± 0.09	0.32 ± 0.15	0.43 ± 0.10	0.43 ± 0.13
	37	1.07 ± 0.29	0.43 ± 0.10	0.30 ± 0.10	0.55 ± 0.21	0.46 ± 0.36	0.36 ± 0.44	0.46 ± 0.15	0.48 ± 0.10	0.55 ± 0.12
500	25	7.99 ± 0.80	6.71 ± 1.20	6.72 ± 0.40	6.96 ± 0.11	6.26 ± 0.40	6.73 ± 0.85	6.30 ± 1.50	8.76 ± 0.73	8.40 ± 1.00
	37	13.30 ± 2.40	7.59 ± 0.20	8.40 ± 0.60	6.94 ± 0.60	4.69 ± 0.80	7.72 ± 0.56	7.30 ± 0.13	8.00 ± 0.72	8.19 ± 0.60

Table 4.27. Nitrate Reductase Activities In The Wild-Type And Variouse Non-Conditional *crn* Mutants.[a]

Strain[c]	Nitrate reductase activity[b]		
	8h	12h	16h
Wild-type	91.91 ± 0.8	67.16 ± 14.6	22.13 ± 6.0
crnA1	60.97 ± 4.2	26.77 ± 3.6	24.10 ± 3.4
crnA688	56.53 ± 2.6	25.74 ± 2.3	21.72 ± 1.8
crnB976	57.20 ± 5.2	31.30 ± 1.4	23.70 ± 2.4
crnB967	56.80 ± 4.2	31.00 ± 2.2	21.40 ± 2.9
crnC561	56.05 ± 10.4	27.22 ± 6.2	21.98 ± 5.3
crnC1056	48.61 ± 4.5	29.03 ± 3.1	19.57 ± 1.1
crnD1116	50.36 ± 2.8	29.20 ± 1.6	23.74 ± 2.7

[a] Mycelia were grown in glucose minimal medium supplemented with nitrate (10 mM) as sole nitrogen source, at 37°C for 8, 12 and 16 hours, as indicated.

[b] Enzyme activities are given as nanomoles of NADPH oxidized per minute per milligram protein. Values are mean ± standard deviation (SD) of three independant grow up experiments.

[c] Representative mutants. The strain number is the original number that was given to the mutant when it was selected on ClO_3^-.

* Assay were carried out at 25°C.

Table 4.28. Nucleotide Changes In *crnA* Mutants.

mutant number	Mutant	Type of mutation	Sequence change/protein change	Affect on the protein
1	*crnA1009*	non-conditional	7 bp deletion frame shift	second last membrane spanning domain and thereafter altered..
2	*crnA1087*	non-conditional	single base pair substitution (G is converted A) gly to glu change.	seventh membrane spanning domain altered by one amino acid.
3	*crnA1*		complete loss of CRNA protein	first membrane spanning domain and thereafter
4	*crnA*517*		no change	altered.
5	*crnA*688*		no change	
6	*crnA*760*		no change	
7	*crnA*775*		no change	
8	*crnA*1025*		no change	

Table 4.29. A Summary Of Phenotypes And Gene Designation Of Identified Genes Revealed In This Study In *Aspergillus nidulans*.

Mutant	Phenotype[a]	Final Gene Designation
crnA	chl-R/ces-S	*crnA*[b]
crnB	chl-R/ces-S	*crnB*[b]
	chl-R/ces-R	*chlA*[c]
	chl-S/ces-S	*cesA*[d]
crnC	chl-R/ces-S	*crnC*[b]
	chl-R/ces-R	*chlA*[c]
	chl-S/ces-S	*cesA*[d]
crnD	chl-R/ces-S	*crnD*[b]

(a) Phenotypes of progeny that came out of outcrosses to the wild-type. **(b)** No recombinant progeny came out of outcrosses between wild-type and *crnA*, *crnB*, *crnC* or *crnD* mutant alleles i.e. all are parental phenotypes no R R or S S recombinant progeny. **(c)** chlorate resistant-caesium resistant recombinant progeny from outcrosses of *crnB* or *crnC* mutant alleles to the wild-type. **(d)** chlorate sensitive-caesium sensitive recombinant progeny revealed from out crosses of *crnB* or *crnC* mutant alleles to the wild-type.

CHAPTER FIVE.

CHARACTERISATION OF NITRITE UPTAKE ACTIVITIES IN *A. nidulans*.

5.1 The Objectives Of This Research Section.

In the alga *Chlamydomonas reinhardtii* three transporter genes have been identified, two nitrate transporters, one nitrate specific system while the other is bispecific which transport both nitrate and nitrite. The third transporter is a nitrite specific system (Quesada *et. al.*, 1994; Galven *et. al.*, 1996). Since nitrite uptake has been studied only in *C. reinhardtii*, the first aim of this part of work was to determine if *crnA1* and the *crn* mutants isolated during the course of this work transport nitrite. Also the nitrite hypersensitive (i.e. *meaB6*, *niiC628* and *tamA105* see introduction) mutants were investigated for nitrite transport. The second aim was to determine if such transporters possess an active transport system or whether nitrite is taken passively. The third aim was to determine if such transporters are specific for either of the ions (i.e. nitrate or nitrite) or bispecific systems taking up both ions. The final aim was to study the effect of different inhibitors on nitrite uptake activity.

5.2 Characterisation Of Nitrite Uptake Activities In *A. nidulans* Wild-Type Strain.

5.2.1 Effect Of pH On Nitrite Uptake Activity.

Wild-type mycelial cells were grown for 16 h at 37°C in glucose minimal medium with urea (5 mM) as the sole nitrogen source. Induction of the transport system by nitrate (10 mM) as the sole inducing nitrogen source was performed by adding nitrate to cells 3 h before harvesting. In nitrite assay media, nitrite uptake rates were determined at 37°C after 21 min of incubation at different pH values (Figure 5.1). Uptake rates were determined in nmol/min/ mg blotted wet weight using either 25 μM (Figure 5.1, Panel A) or 3 mM nitrite (Figure 5.1, Panel B) in 20 mM phosphate buffer. The nitrite uptake values obtained (Figure 5.1, Panel A) at 37°C indicated that the nitrite uptake rate for wild-type cells, was optimal (approx 0.052 nmol/min/mg) at pH 6.5, when 25 μM nitrite was present. When 3 mM (Figure 5.1, panel B) nitrite were present in the assay system, the maximum uptake rate of nitrite was 4.5 nmol/ min/mg again at pH 6.5. Such data suggests that maximum nitrite uptake activities were achieved at pH 6.5.

5.2.2 Effect Of Temperature On Nitrite Uptake Activity

Wild-type cells were grown and induced as outlined in section 5.2.1. Nitrate induced cells were washed and transferred to nitrite assay minimal medium containing either 25 μM or 3 mM

nitrite. Nitrite uptake values were determined after incubation 21 min at pH 6.5 and at a range of incubation temperature (i.e. 25°C to 38°C, Figure 5.2). Nitrite uptake rates presented in Figure 5.2 Panel A showed that nitrite uptake capacity was maximum (i.e. 0.047 nmol/min/mg blotted wet) at 37°C when 25 μM nitrite were used. However when 3 mM nitrite were used the maximum nitrite uptake rate was 3.76 nmol/min/mg blotted wet (Figure 5.2, panel B) at the same temperature. These data indicates that the optimum temperature for the achievement of maximum nitrite uptake rate was 37°C.

5.2.3 Regulation Of Nitrite Uptake Activity In Young Wild-Type Cells.

The effect of different nitrogen sources (i.e. ammonium, nitrite or nitrate) on the regulation i.e. induction or repression of nitrite uptake expression, has been studied in young wild-type cells grown for 8 h at 37°C. Mycelial cells were grown in the presence of ammonium, nitrite or nitrate (10 mM) for either 100 min or 3 h before harvesting then washed and transferred to assay media containing 25 μM nitrite. Nitrite uptake values were determined in a time-course with intervals each of 3 min up to 21 min.

Data presented in Figure 5.3, Panel A indicates that after 100 min induction, nitrate acted as the best inducer. In this respect the uptake capacity of nitrite after 21 min incubation was approximately 0.20 nmol/min/mg blotted wet weight. This uptake

capacity was reduced to approximately 0.12 nmol/min/mg when nitrite acted as the inducer for the same incubation period (i.e. 21 min). The uptake capacity was further reduced to 0.073 nmol/min/mg when ammonium served as the sole nitrogen source for 100 min before harvesting.

Additionally, Figure 5.3, Panel B shows that after 3 h induction, nitrate remained the best inducer. The nitrate uptake capacity after 21 min incubation in the assay medium was approximately 0.37 nmol/mg blotted wet weight. When nitrite was the inducer this uptake capacity was reduced to approximately 0.18 nmol/mg, whereas, the capacity was 0.07 when ammonium was used.

Data presented in Figure 5.3 indicate that nitrite uptake capacity was the best when wild-type cells were induced by nitrate 3 h instead of 100 min before harvesting. Nitrite acted as an inducer but to a lesser extent as compared with nitrate and this may be due its slight toxicity to cells (i.e. Nitrite is generally considered to be toxic to cells. In contrast, ammonium acted as a repressor rather than an inducer for uptake expression.

5.2.4 Regulation Of Nitrite Uptake Activity In Older Wild-Type Cells.

Wild-type cells of 16 h incubation were treated with ammonium, nitrite or nitrate as an

inducer or repressor for uptake system expression 100 min or 3 h before harvesting. In addition both ammonium and nitrate were used together in a 3 h induction period.

Data presented in Figure 5.4, panel A shows that after 100 min induction nitrate was the best inducer for the nitrite uptake system, where the uptake capacity after 21 min incubation in the assay medium, was approximately 0.35 nmol/mg. This capacity was reduced to approximately 0.18 nmol/mg when nitrite was used under the same induction period (i.e. 100 min before harvesting). However, activity ranged between 0.07 to 0.1 nmol/mg when ammonium was used.

Moreover, the nitrite uptake data obtained after 3 h induction (Figure 5.4, panel B) indicate again nitrate was the optimum inducer for the uptake system where the nitrite uptake capacity after 21 min incubation in the assay medium, was approximately 0.95 nmol/mg. However, when nitrite was used this capacity of taking up nitrite was reduced to approximately 0.6 nmol/mg blotted wet weight. When ammonium was used, this activity was minimised to approximately 0.075 nmol/mg and when both ammonium and nitrate were present nitrite uptake activity ranged between 0.2 nmol/mg to 0.05 nmol/mg. Data presented in Figure 5.3 and 5.4 indicate that nitrite uptake capacity in older but not in younger cells was highly influenced after 3 h induction by nitrate i.e. the uptake value in older cells was 0.95 nmol/mg compared to 0.2 nmol/mg in younger

cells under the same induction conditions. However, nitrite itself was acting as a second inducer at both cell ages (i.e. 8 h and 16 h old) and induction periods.

With nitrite induction, the highest uptake activity was achieved after 3 h induction in older cells (i.e. 0.6 nmol/mg blotted wet weight and this value was twice of that obtained with older cells induced for 100 min by nitrate. These results indicate that ammonium at least at low concentrations may act as a strong repressor of the nitrite transport system even in the presence nitrate.

5.2.5 Inhibition Of Nitrite Uptake Activity In Older Wild-Type Cells.

After the determination of nitrite uptake Km value for wild-type (i.e. 1004 ± 116 μM) I studied the effect of different concentrations of potential inhibitors on the activity of nitrate induced nitrite uptake in older wild-type cells. Mycelial cells were grown for 16 h and induced by nitrate 3 h before harvesting then transferred to assay media containing 3 mM nitrite (i.e. 3 x the Km value). Data presented in Figure 5.5, panel A shows the effect of a range of ammonium concentrations on nitrite uptake capacity.

The results show that wild-type cells assayed in the presence of 3 mM nitrite, but in the absence of ammonium, have nitrite uptake capacity of approximately 80 nmol/mg blotted wet weight after 21 min incubation in assay media. However, all different

ammonium concentrations with the exception of 10 mM reduced such activity to approximately 50 to 55 nmol/mg. In contrast, this capacity was found to be reduced to only 67 nmol/mg when 10 mM were used. Data presented in Figure 5.5, panel B indicate that when nitrate, at a concentration of 0.5, 1, or 5 mM, was used in the assay media, nitrite uptake capacity was reduced from 80 nmol/mg to approximately 50 nmol/mg. However, this capacity was reduced to 45 nmol/mg when 10 mM nitrate were used whereas, 20 mM nitrate reduced the uptake capacity from 80 to approximately 63 nmol/ mg.

Furthermore, the results in Figure 5.5, panel C show that nitrite uptake activity is affected by the presence of chlorate. The nitrite uptake rate falls to 20 nmol/mg when 20 mM chlorate were used to approximately 40 nmol/mg when 10 mM chlorate were used. The results indicated that different concentrations of certain inhibitors used, showed approximately the same effect on the reduction of nitrite uptake capacity.

When 3 mM nitrite were used in the assay media, ammonium did not show the strong inhibitory effect on the activity seen at 25 μM nitrite. Ammonium appears to act as a strong repressor for the uptake system, not as an inhibitor. In contrast, chlorate, at different concentrations used, acted as a strong inhibitor on nitrite uptake activity.

5.3 Characterisation Of Nitrite Uptake Activity In *A. nidulans* Mutants.

Nitrite uptake activity was investigated in the *crnA1* mutant as well as representative from each of the newly identified *crn* genes. Additionally, three more *Aspergillus* mutants that showed hypersensitivity to nitrite (i.e. may be involved in nitrite uptake and may have nitrite transport abnormalities) were included in this part of the work. Mycelial cells were grown for 16 h at 37°C before harvesting. After harvesting, the cells were transferred to the nitrite assay media and nitrite uptake values were determined during time-course intervals of 3 min up to 21 min incubation at 37°C in the assay media.

5.3.1 Uptake Activity Of Nitrite (25 μM) In Older Cells.

The activity of the nitrate induced nitrite uptake system in wild-type and various mutant cells was determined in the presence of 25 μM nitrite and at time-course intervals each of 3 min up to 21 min (Figure 5.6). In panel A, after 21 min incubation in the assay medium mycelial cells of *meaB6* strain showed nitrite uptake capacity of approximately 1.25 nmol/mg blotted wet weight, as compared to approximately 0.45 nmol/mg obtained by wild-type cells. Mycelial cells of the strain *niiC628* showed an uptake activity of approximately 1.15 nmol/mg, whilst *tamA105* cells showed an activity of approximately 1 nmol. mg as compared to 0.45 nmol/mg of the wild-type cells under the same assay conditions.

In a separate experiment (Figure 5.6, panel B) nitrite uptake activity in mutant *crnC1056* mutant cells was approximately 1.25 nmol/mg as compared to 0.7 nmol/mg of wild-type cells. Nitrite uptake capacity in mutant *crnB967* cells was approximately 1.18 nmol/mg whilst that of mutant *crnD1116* was 0.95 nmol/mg. In contrast, such activity was reduced in mutant *crnA1* to approximately 0.62 nmol/mg. These results indicate that strains *crnC1056*, *crnB967*, *meaB6* and *niiC628* mutants showed approximately the same nitrite uptake activity (i.e. approx 1.1 to 1.2 nmol/mg), whereas, *crnD1116* and *tamA105* mutants showed an activity of approximately 1 nmol/mg blotted wet weight mycelia. In contrast, *crnA1* mutant cells showed the lowest activity even slightly lower than that of the wild-type cells (i.e. approx 0.65 nmol/mg blotted wet weight).

5.3.2 Effect Of 1 mM Ammonium On Nitrite (25 μM) Uptake Activity Of Older Cells.

In the presence of 1 mM ammonium nitrite uptake capacity of *meaB6* cells after 21 min incubation in the assay media was approximately 1.1 nmol/mg. blotted wet weight as compared to 0.32 nmol/mg obtained by wild-type cells (Figure 5.7, panel A). Uptake capacity of *niiC628* cells was approximately 1.2 nmol/mg whereas, such activity was reduced to approximately 0.52 nmol/mg for *tamA105* mutant cells. Nitrite uptake capacity of *crnA1* cells was approximately 0.62 nmol/mg in the presence of ammonium (1 mM) as compared to approximately 0.3 nmol/mg of wild-type cells. Such

activity was increased to approximately 1.2 nmol/mg for mutant *crnB967* and mutant *crnC1056* whereas, this activity was reduced to approximately 0.68 nmol/mg for mutant *crnD1116* cells (Figure 5.7, panel B).

Comparison of these results and those presented in Figure 5.6 (i.e. the absence of ammonium) indicate that 1 mM ammonium as an inhibitor of nitrite uptake did not affect nitrite uptake activity in mycelial cells of mutants *meaB6*, *niiC628*, *crnA1*, *crnB967* or *crnC1056*. Such activity was slightly reduced by ammonium in *crnD1116* mutant cells. In contrast, *tamA105* mutant cells showed an approximately 50% reduction in activity in the presence of ammonium.

5.3.3 Uptake Activity Of 10 μM Nitrite In Older Cells Of Wild-Type And Mutant Cells.

Mycelial cells of wild-type or various mutant cells showed nitrite uptake activity at very low concentration of nitrite (i.e. 10 μM) (Figure 5.8). Both *niiC628* or *tamA105* mutant cells showed a nitrite uptake capacity of approximately 0.5 nmol/mg as compared to 0.31 nmol/mg for wild-type cells grown, under the same assay conditions (Figure 5.8, panel A). Such activity was slightly reduced in *meaB6* cells (i.e. 0.44 nmol/mg). Nitrate induced nitrite uptake system was approximately 0.5 nmol/mg for either *crnB967* or *crnD1116* mutant cells (Figure 5.8, panel B). However, *crnC1056* mutant cells showed an activity of

0.48 nmol/mg, whereas, such activity was approximately 0.36 nmol/mg for *crnA1* mutant cells as compared to 0.3 nmol/mg for the wild-type. Data presented in Figure 5.8 indicate that all mutant cells (with the exception of *crnA1* which showed approximately the same activity as the wild-type) showed approximately double activity of nitrite uptake as compared to wild-type in the presence of 10 μM nitrite. This is the same pattern seen at 25 μM nitrite (Figure 5.6).

5.3.4 Inhibition Of Nitrite Uptake (10 μM) Activity By Nitrate (50 μM) In Older Cells Of Wild-Type And Mutant Strains.

The effect of nitrate (50 μM) on the activity of the nitrate induced nitrite (10 μM) uptake system in the wild-type and various mutants indicate that nitrate did not affect nitrite uptake activity as compared to the activity carried out in the absence of nitrate. Such data showed that the mutant *niiC628* showed a nitrite uptake capacity of approximately 0.5 nmol. mg as compared to 0.27 nmol/mg of wild-type. However, either *tamA105* or *meaB6* cells showed an activity of approximately 0.46 nmol/mg. Mycelial cells of mutants *crnB967*, *crnC1056* or *crnD1116* showed nitrite uptake activity of approximately 0.5 nmol/mg blotted wet weight (Figure 5.9, panel B). Such activity in *crnA1* cells was approximately 0.3 nmol/mg as compared to 0.27 nmol/mg of the wild-type. Such data presented in Figure 5.9 indicate that all mutant cells showed an uptake activity of nitrite which is higher than that in

the wild-type cells. Additionally such activity was not affected by the presence of 50 μM nitrate when compared with in the absence of nitrate.

5.3.5 Inhibition Of Nitrite (10 μM) Uptake Activity By Chlorate (1.5 mM) In Older Cells Of Wild-Type And Mutant Strains.

The effect of chlorate (i.e. the toxic analogue of nitrate) on the activity of nitrate induced nitrite uptake system is shown in Figure 5.10. Such data indicated that mycelial cells of mutant *niiC628* showed the highest activity of taking up nitrite in the presence of chlorate (1.5 mM) where such activity was approximately 0.53 nmol/mg after 21 min incubation in the assay medium (Figure 5.10, panel A). Such activity was approximately 2-fold more than that obtained by wild-type cells. However, mycelial cells of either *meaB6* or *tamA105* showed an activity in the range of 0.4 to o.45 nmol/mg. Data in Figure 5.10, panel B indicate that either *crnB967* or *crnD1116* showed an uptake activity of nitrite of approximately 0.48 nmol/mg. *crnC1056* cells showed an activity of approximately 0.4 nmol/mg, Activity in *crnA1* cells was 0.3 nmol/mg as compared with 0.12 nmol/mg of the wild-type Figure 5.10. All mutant cells showed nitrite uptake activity higher than that of the wild-type even in the presence of chlorate (1.5 mM). These data showed also that such nitrite activity was not affected by the inhibitors (as compared to activity found in the absence of such inhibitors) (Figure 5.8).

5.3.6 Kinetic Parameters Of Nitrite Transport In *A. nidulans.*

As a first attempt of studying nitrite transport system in *A. nidulans* it was decided to study the nitrite uptake activity at low nitrite concentrations, since such substrate is slightly toxic to the living cells. However, since no previous nitrite uptake studies have been carried out in *Aspergillus nidulans* so it was decided to determine the kinetic parameters (i.e. Km and Vmax values) of nitrite transport and to study the uptake activity and the effect of different inhibitors on such transport system.

The kinetics of nitrite uptake was studied using both the initial rate method and the analytical determinations (section 2.5.2). Initial rate of nitrite uptake at different nitrite concentrations (i.e. 10, 50, 100, 500, 1000, 2500, 5000, 7000 and 10,000 μM) was estimated as the mean velocity over a period of 2 min. This short period of time is assumed to be the initial velocity corresponding to the mean substrate concentration in the same period.

Kinetic parameters (i.e. Km and Vmax values) were calculated using the programme ENZ FITTER designed for the IBM computers. For the analytical determinations nitrite uptake values in terms of nmol/mg blotted wet weight were determined by measuring the disappearance of the substrate from the assay medium at time-course intervals of 3 min up to 21 min. The absorbance obtained was applied to an equation of nitrite standard curve (Appendix 2).

Data presented in Table 5.1 showed the kinetic parameters of nitrite transport for wild-type and various mutant strains of *A. nidulans*. Such data were determined by graphical representation of Michaelis-Menten equation using the programme ENZ FITTER. A representative graphical representation for the *meaB6* mutant strain is shown in Figure 5.11. These kinetic parameters indicate that Km values obtained for nitrite transport in the wild-type strain was approximately similar to those obtained in *crnA1*, *crnB967* and *crnD1116* mutant strains (i.e. around 1,000 μM). The nitrite hypersensitive mutants (i.e. *meaB6*, *niiC628* and *tamA105*) showed lower Km values i.e. 588, 688 and 770 μM respectively (Table 5.1). The V max value for nitrite transport in wild-type strain was the lowest as compared to all mutant strains studied (Table 5.1). The mutants *crnA1* and *crnB967* showed the highest Vmax values.

Depending on these kinetic parameters, it was decided to study nitrite uptake activity in wild-type and various mutant strains in the presence of 3 mM (i.e. 3 X the Km value of wild-type) nitrite in the assay media and study the effect of different inhibitors (i.e. ammonium, nitrate or chlorate) at a concentration of 10 mM on such activity. These results shown in Figure 5.12, panel A indicate that in the presence of 3 mM nitrite in the assay media mycelial cells of *meaB6* , *niiC628* or *tamA105* mutant strains showed nitrite uptake activity of approximately 107 nmol/mg, 95 nmol/mg and 87 nmol/mg respectivily as compared to 77 nmol/mg obtained by the wild-type cells.

Data in Figure 5.12, panel B indicate that the activity of the nitrate induced nitrite uptake system in either *crnC1056* or *crnD1116* mutant strains was approximately 95 nmol/mg as compared to approximately 70 nmol/mg in the wild-type strain. The *crnA1* or *crnB967* mutants showed a wild-type activity under the same assay conditions. The three nitrite hypersensitive mutants (i.e. *meaB6*, *niiC628* and *tamA105*) in addition to *crnC1056* and *crnD1116* mutants showed a higher nitrite uptake activity as compared to the wild-type (Figure 5.12). However, the *tamA105*, *crnA1* or *crnB967* strains showed the wild-type activity or slightly higher activity.

A comparison of these activities in nitrite uptake with the activities obtained when low concentrations of nitrite i.e. 10 μM (Figure 5.8) or 25 μM (Figure 5.6) indicated the following. First, in all concentrations of nitrite used, all mutant strains were more active in taking up nitrite than wild-type with the exception of *crnA1* mutant which showed a wild-type activity or slightly higher. Second, mycelial cells of *crnB967* mutant showed a higher nitrite uptake activity at the lower concentrations however, such activity reduced to wild-type level when higher (i.e. 3 mM) concentration of nitrite was used.

5.3.7 Inhibition Of Nitrite (3 mM) Uptake Activity By Nitrate (10 mM) In Older Cells Of Wild-Type And Mutant Strains.

The effect of nitrate (10 mM) on the activity of the nitrate induced nitrite uptake system is shown in Figure 5.13. Such data in Figure 5.13, panel A indicate that both nitrite hypersensitive mutants *meaB6* and *tamA105* showed nitrite uptake capacity which is slightly higher (i.e. approx 77 nmol/mg) than that of the wild-type (i.e. approx 70 nmol/mg). However, strain *niiC628* showed a wild-type activity of nitrate transport. Both *crnC1056* and *crnD1116* mutants showed an activity of approximately 90 nmol/mg as compared to 62 nmol/mg of the wild-type (panel B of Figure 5.13).

However, such activity was approximately 68 nmol/mg in *crnB967* cells whereas, *crnA1* mutant showed a wild-type activity. Such results would indicate that nitrate as an inhibitor for nitrite has reduced the nitrite uptake activity in the hypersensitive strains to a wild-type level as compared to the activity in the absence of the inhibitor (Figure 5.12). In contrast, nitrate was not affecting nitrite uptake activity in *crn* mutants where such mutants showed the same activity in the presence or in the absence of nitrate. Furthermore, a comparison of these data in Figure 5.13 with that in Figure 5.9 (i.e. nitrate at a concentration of 50 μM) was used with 10 μM nitrite) indicate that nitrate at a concentration of 50 μM was not affecting nitrite uptake of 10 μM.

5.3.8 Inhibition Of Nitrite (3 mM) Uptake Activity By Chlorate (10 mM) In Old Cells Of Wild-Type And Mutant Cells.

The effect of chlorate (10 mM) on the activity of the nitrate induced nitrite (3 mM) uptake system in older cells is presented in Figure 5.14. Such data indicate that all studied mutants show an uptake activity of nitrite which is higher than that of the wild-type. Data in Figure 5.14, panel A showed that the *meaB6* mutant strain had an activity of approximately 72 nmol/mg as compared to 40 nmol/mg of the wild-type. However, the *niiC628* strain showed an activity of approximately 64 nmol/mg, whereas, *tamA105* strain showed an activity of 55 nmol/mg. Additionally, data in Figure 5.14, panel B indicate again that both *crnC1056* and *crnD1116* showed the highest activity i.e. approximately 74 nmol/mg as compared to 47 nmol/mg of that of the wild-type. However, *crnB967* showed an activity which is slightly higher than the wild-type i.e. approximately 60 nmol/mg whereas, *crnA1* showed a wild-type activity. A comparison of these data with that in the absence of any inhibitor (Figure 5.12) indicate that chlorate has reduced nitrite uptake activity to approximately 50% in the nitrite hypersensitive mutants. However, 25% reduction in such activity was observed in both *crnC1056* and *crnD1116* mutants. Furthermore, a comparison of the data presented in Figure 5.13 with that in Figure 5.10 (i.e. where 1.5 mM chlorate was used with 10 μM nitrite) indicate that chlorate was showing a strong effect on mutant strains as compared to wild-type. These comparisons lead to

the suggestion that chlorate was affecting strongly nitrite uptake in the strains studied.

5.3.9 Inhibition Of Nitrite (3 mM) Uptake Activity By Ammonium (10 mM) In Older Cells Of Wild-Type And Mutant Strains.

The effect of ammonium on the activity of the nitrate induced nitrite uptake system in older cells of wild-type and mutant strains is presented in Figure 5.15. Such data indicate that *meaB6* strain showed the highest nitrite uptake activity i.e. approximately 85 nmol/mg as compared to 65 nmol/mg of the wild-type. The *niiC628* strain showed an activity of approximately 77 nmol/mg, while, *tamA105* showed an activity of 62 nmol/mg. Furthermore, data in Figure 5.15, panel B indicate that *crnC1056* showed the highest activity i.e. 85 nmol/mg as compared to 67 nmol/mg of the wild-type. *crnD1116* mutant cells showed an activity of 75 nmol/mg, whereas, *crnB967* and *crnA1* showed wild-type activity of nitrite uptake.

A comparison of these data with that presented in Figure 5.12 i.e. in the absence of any inhibitor indicate that ammonium has reduced nitrite uptake activity by approximately 1/5 in the hypersensitive mutants and both *crnC1056* and *crnD1116* mutants. Such activity was slightly reduced in *crnA1* and *crnB967* mutants. Furthermore, a comparison of the activity shown in Figure 5.15 and that in Figure 5.7 (i.e. when 1 mM ammonium was used with 25 μM

nitrite) indicate that both nitrite hypersensitive mutants (i.e. *meaB6* and *niiC628*) in addition to *crnB967* and *crnC1056* mutants showed 4 to 5 fold increase in the activity as compared to wild-type (Figure 5.7) when 1 mM ammonium was used with 25 μM nitrite . However, when ammonium at a concentration of 10 mM (i.e. Figure 5.15) was used with 3 mM nitrite the uptake activity of nitrite in these strains was slightly higher than that of the wild-type. These findings indicate that ammonium was not inhibiting the uptake of nitrite in all strains included in this study.

5.3.10 Inhibition Of Nitrite Uptake By Different Nitrate Concentrations.

The effect of various nitrate concentrations (i.e. 10, 50, 100, 300, 600, 1000, 5000, 10,000 and 20,000 μM) on the nitrate induced nitrite uptake system was studied in wild-type and various mutant strains (Figure 5.16). Initial nitrite uptake rates (i.e. after 2 min) were determined at the nitrite concentrations used (i.e. 10, 50, 100, 500, 1000, 2500, 7000 and 10,000 μM) in the absence or the presence of nitrate (Figure 5.16). Such results show that nitrate as an inhibitor has reduced nitrite uptake activity in wild-type and all mutant strains included in this study particularly when high nitrate concentrations (i.e. 20 mM) was used.

When 20 mM nitrate was used with 10 mM nitrite, uptake rate of nitrite in wild-type strain was reduced from approximately 18 to 5 nmol/min/mg blotted wet weight (Figure 5.16). In *crnA1* strain such activity was reduced from approximately 32 to 14

nmol/min/mg. Nitrite uptake rate in mutant *crnB967* was reduced from 27 to 7 nmol/min/mg. In the *crnC1056* mutant 20 mM nitrate has reduced the nitrite uptake rate from 21 to 3 nmol/min/mg. The activity of *crnD1116* strain was reduced from 22 to 6 nmol/min/mg. In the nitrite hypersensitive mutant *meaB6* nitrite uptake rate was reduced from approximately 23 to 4 nmol/min/mg. In *niiC628* such activity was reduced from 24 to 4 nmol/min/mg.Finally, *tamA105* activity was reduced from 21 to 8 nmol/min/mg blotted wet weight.

5.4 Discussion.

Nitrite uptake data obtained in this section of the work using low (i.e. 25 μM or less) and high (i.e. 3 mM) nitrite concentrations may suggest that entry of nitrite into *A. nidulans* cells is mediated by an active transport system, rather than just passive diffusion of the substrate through cell membrane.

Nitrite uptake activity was observed at low nitrite concentrations in the assay media (i.e. 25 μM) in either young or old wild-type cells grown in ammonium for 100 min or 3 h before harvesting indicate that such activity was blocked by ammonium. Additionally, when *A. nidulans* wild-type cells were supplied with both nitrate and ammonium, nitrite uptake was prevented. This inhibition could be due to one of several possibilities: First, ammonium was acting as a repressor which repressed the synthesis of the transport protein. Second, ammonium inhibits the activity of

the transport system. Third, since ammonium is known as a repressor for the enzymatic steps (i.e. nitrite reduction and nitrate reduction) of the nitrate assimilation pathway such repression may indirectly inhibit nitrite conversion and ultimately prevents the uptake process. In contrast, nitrate or nitrite were acting as inducers for such transport system, where nitrate was the best inducer over nitrite and this could be related to the slight toxicity of nitrite.

Nitrite uptake activity increased approximately 3-fold when wild-type cells were induced by nitrate or nitrite for 3 h rather than 100 min. Furthermore, nitrite uptake activity in older wild-type cells was much higher as compared to that of younger cells. Clearly, nitrite uptake activity in older wild-type cells was doubled as compared to the younger cells when such cells were induced by nitrate 100 min before harvesting. Additionally, there was a 3-fold increase in the activity of the older wild-type cells compared to younger cells when the induction was performed for 3 h. Older wild-type cells are more active in nitrite uptake than younger cells. Such a result may suggest that there are two transport proteins one which is specific for transport in younger cells and the other in older cells. Such latter suggestion would fit with that suggest previously by (Brownlee and Arst, 1983) for nitrate uptake.

Additionally, all putative nitrate transport mutant strains (i.e. *crn* mutants) in addition to the nitrite hypersensitive strains (i.e. *meaB6*, *niiC628* and *tamA105*) showed nitrite uptake activity.

The *crnA1* mutant showed the lowest nitrite uptake activity as compared to other *crn* mutants or the nitrite hypersensitive strains included in this study. Moreover, *crnC1056* and *crnD1116* in addition to *meaB6* and *niiC628* strains showed the highest nitrite uptake activity with all nitrite concentrations used higher than wild-type.

These results suggested that both nitrite hypersensitive mutants were transporting nitrite effectively. Both *crn* genes i.e. *crnC* and *crnD* might encode a bispecific transport system which transports both ions (i.e. nitrate and nitrite) effectively. In contrast, both *crnA* and *crnB* genes could be a nitrate specific transporters that did not transport nitrite effectively as compared to other strains included in the research section, since such mutants possess a wild-type activity of nitrite transport.

Competition experiments with ammonium, nitrate or its toxic analogue chlorate on the nitrite transport by wild-type and mutant strains has been studied. As shown in Figures 5.7 and 5.15 ammonium showed slight effect on nitrite uptake activity but it did not block such activity completely. More specifically, when ammonium was used at a concentration of 1 mM, with 25 μM nitrite or 10 mM , with 3 mM nitrite the results obtained indicate that although ammonium acted as a repressor for the synthesis of the transport system but, since such protein is made ammonium showed slight affect on the activity but it did not block such activity

completely. Additionally, both nitrate and chlorate also showed slight effect on nitrite activity but none of these ions has blocked the activity.

Nitrite uptake inhibition by nitrate was also studied by analysing the effect of increased nitrate concentrations on nitrite uptake. Nitrate concentrations up to 20 mM strongly affected nitrite uptake rates of the strains studied. The results could suggest the existence of bispecific transporter for both nitrate and nitrite, where nitrate competes (i.e. competative inhibition) with nitrite at the uptake level. More clearly, the observed inhibition by nitrate suggests the presence of common active sites on the transporters for both ions, i.e. the existence of specific nitrite transporters is uncertain.

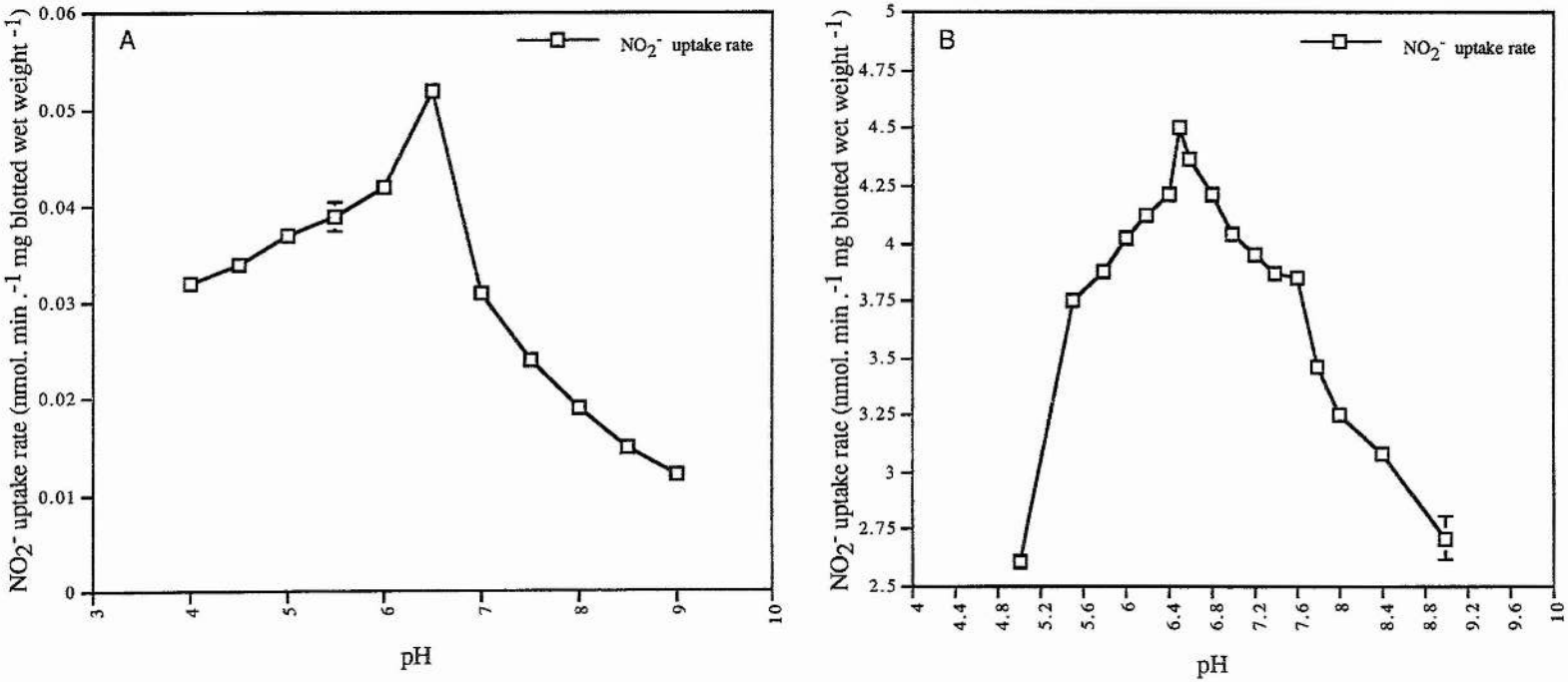


Figure 5.1. The Effect Of pH On Nitrite Uptake Activity In The Wild-Type. Mycelia were grown for 16 h at 37°C, in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells) 1 h before and transferred to a new media with nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium. Rates were measured at 37°C after 21 min of incubation at different pH as indicated above. Rates were determined in nmol.min $^{-1}$ mg $^{-1}$ by using either 25 µM **(panel A)** or 3 mM **(panel B)** sodium nitrite in 20 mM phosphate (as potassium salt) buffer, at the pH indicated in the presence of 1% glucose. Values are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified on the graph , where the maximum standard deviation was no greater than **(Panel A)** ± 0.002, **(Panel B)** ± 0.094.

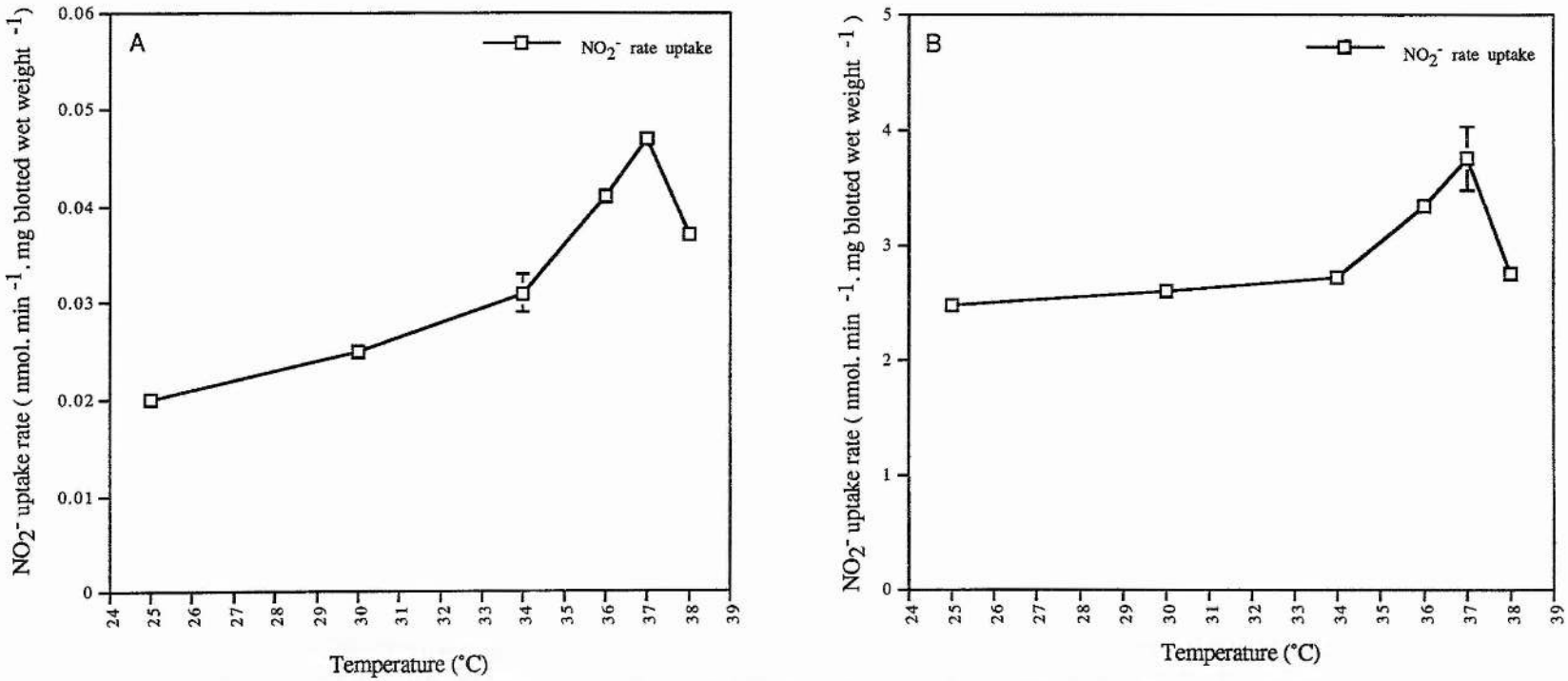


Figure 5.2. The Effect Of Temperature On Nitrite Uptake Activity In The Wild-Type. Mycelia were grown for 16 h at 37°c, in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing either 25 µM or 3 mM sodium nitrite. Rates were measured at pH 6.5 after 21 min of incubation at the different temperatures indicated above. Rates were determined in nmol.min $^{-1}$.mg $^{-1}$. Values are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified on the graph, where the maximum standard deviation was no greater than **(Panel A)** ± 0.002, and **(Panel B)** ± 0.027.

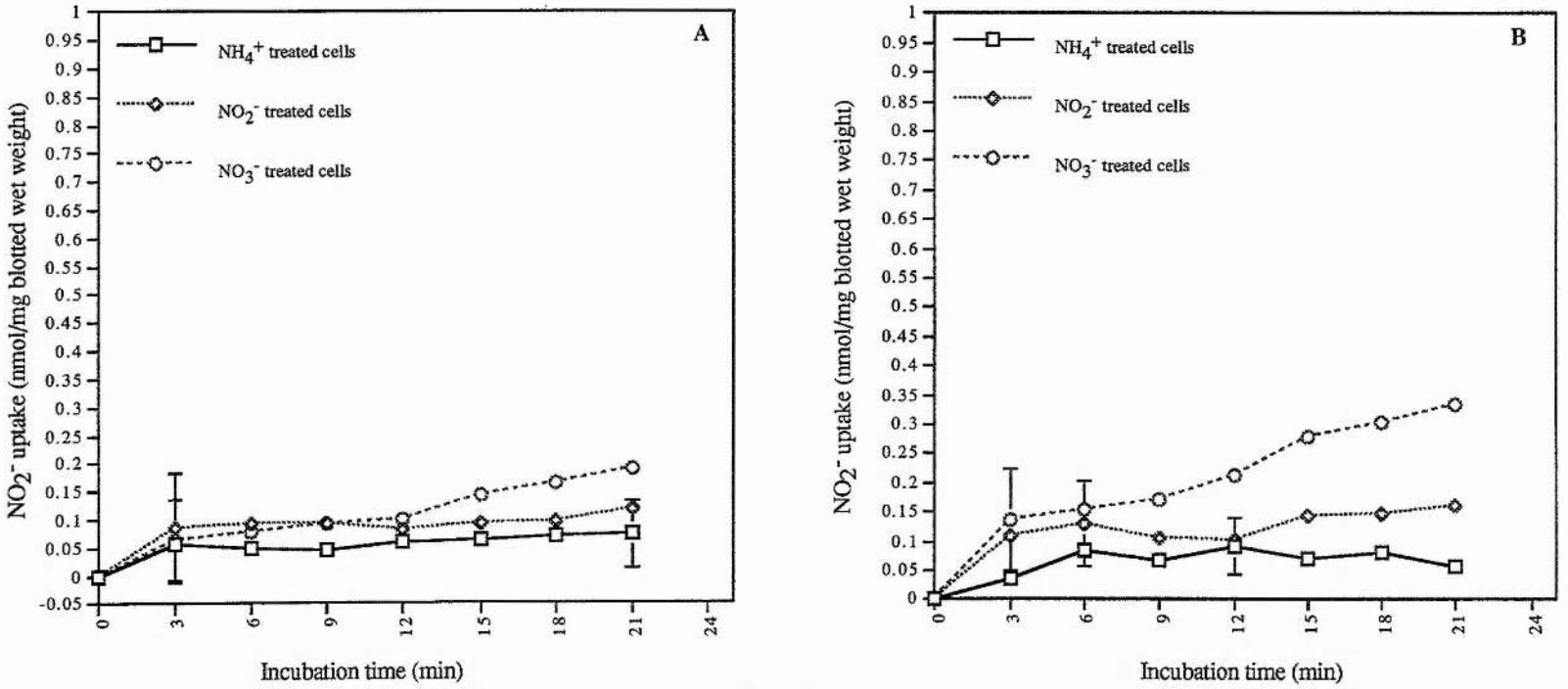


Figure 5.3. Regulation Of Nitrite (25 μM) Uptake Activity In Younger Wild-Type Cells. The effect of various nitrogen sources on the induction or repression of nitrite uptake system in young wild-type cells. Mycelia were grown for 8 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with ammonium, nitrite, or nitrate (all 10 mM) as sole nitrogen source, 100 min **(Panel A)** or 3 h **(Panel B)** before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (25 μM). Uptake values were measured at the indicated times, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than: ammonium ± 0.059; nitrite ± 0.096; nitrate ± 0.075. In **Panel B** the maximum standard deviation was no greater than: ammonium ± 0.049; nitrite ± 0.074; nitrate ± 0.086.

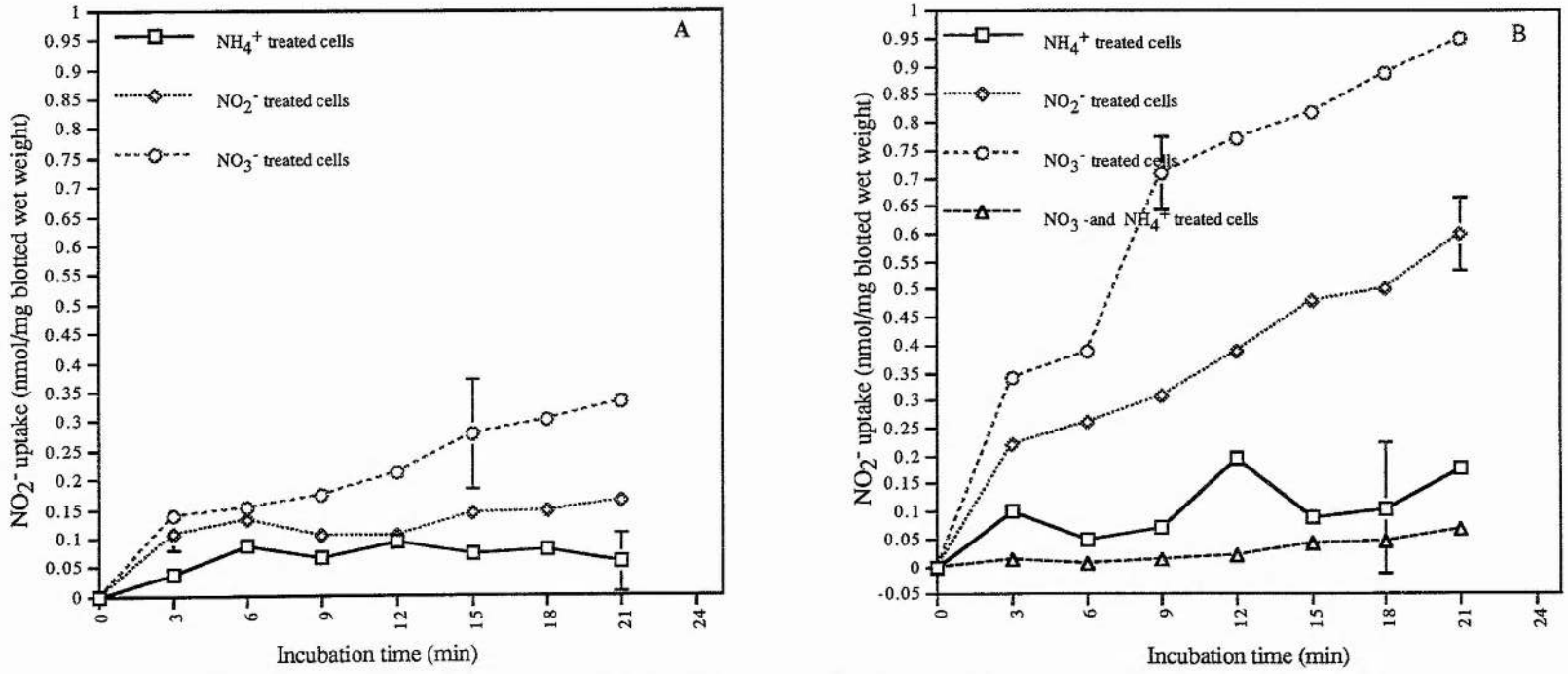


Figure 5.4. Regulation Of Nitrite (25 µM) Uptake Activity In Older Wild-Type Cells. The effect of various nitrogen sources , on the induction or repression of nitrite uptake system in older wild-type cells. Mycelia were grown for16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with ammonium, nitrite, or nitrate (all 10 mM) as sole nitrogen source, 100 min **(panel A)** or 3 h **(panel B)** before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (25 µM). uptake values were measured at the indicated times, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than: ammonium, ± 0.051; nitrite, ± 0.028; nitrate, ± 0.093. In **Panel B** the maximum standard deviation was no greater than: ammonium, ± 0.12; nitrite, ± 0.066; nitrate, ± 0.066.

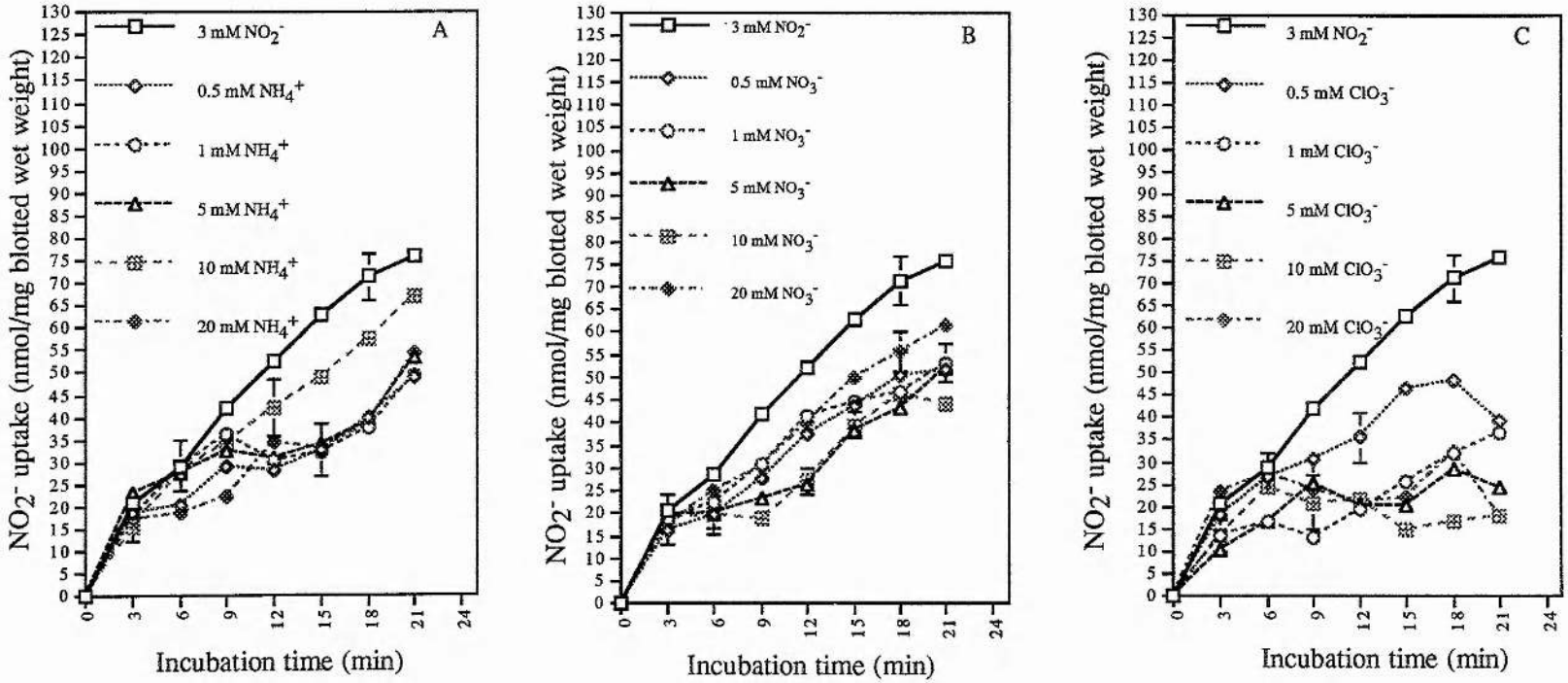


Figure 5.5. Inhibition Of Nitrite (3 mM) Uptake Activity In Older Wild-Type Cells. The effect of various ammonium, nitrate, and chlorate concentrations on the activity of the nitrate induced nitrite uptake system in older wild-type cells. Mycelia were grown for16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with **(panel A)** ammonium, **(panel B)** nitrate, or **(panel C)** chlorate at the concentrations indicated above as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (3 mM). uptake values were measured at the indicated times, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **all Panels** was no greater than: nitrite alone, ± 5.2; **(Panel A)** different ammonium concentrations, ± 6.3; **(Panel B)** different nitrate concentrations, ± 5.6; **(Panel C)** different chlorate concentrations ± 6.1.

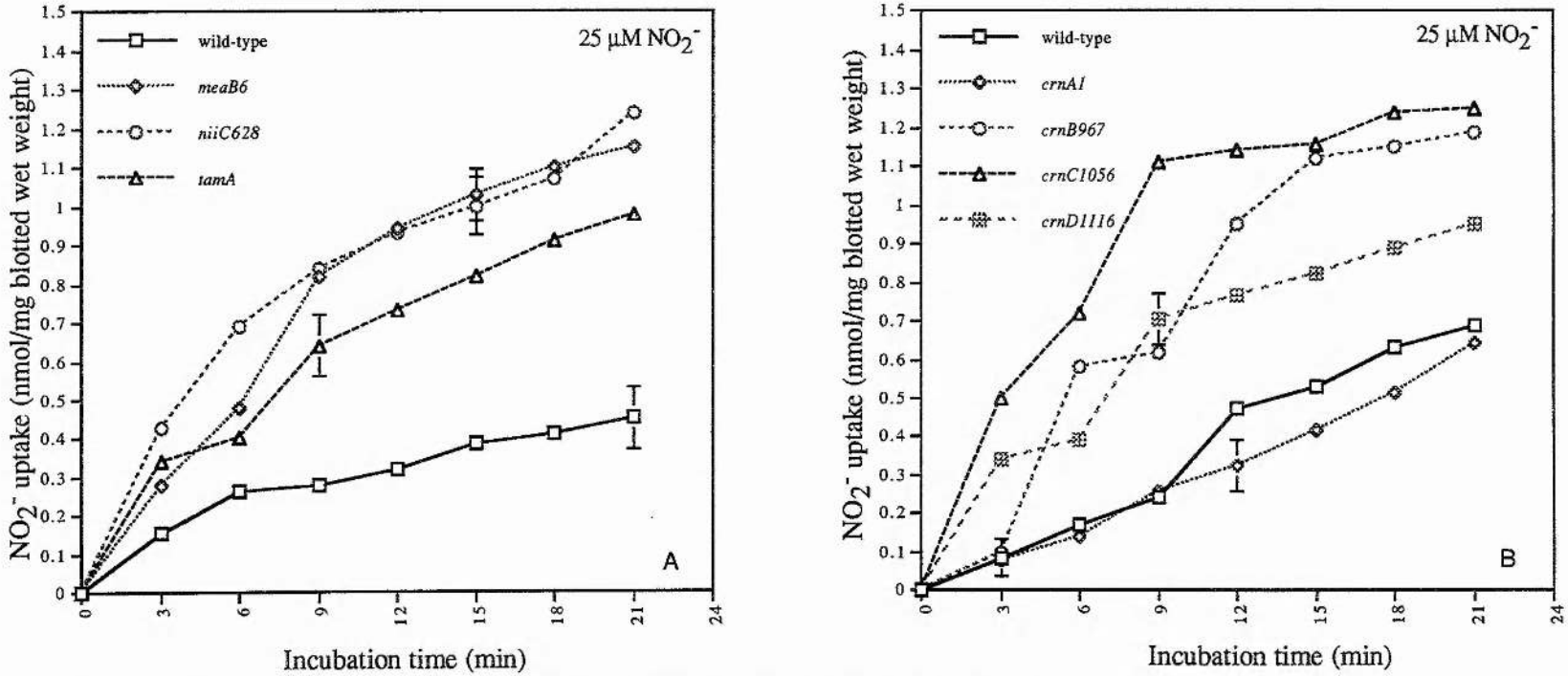


Figure 5.6. Uptake Activity Of Nitrite (25 µM) In Older Cells. The activity of the nitrate induced nitrite uptake system in wild type and various mutant cells. Mycelia were grown for16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with sodium nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (25 µM). Uptake values were measured at the indicated times. Values are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than: wild-type, ± 0.079; *meaB6*, ± 0067; *niiC628*, ± 0.075; *tamA105*, ± 0.08. In **Panel B** the maximum standard deviation was no greater than: wild-type, ± 0.048; *crnA1*, ± 0.068; *crnB967*, ± 0.02; *crnC1056*, ± 0.0.026; *crnD1116*, ± 0.066.

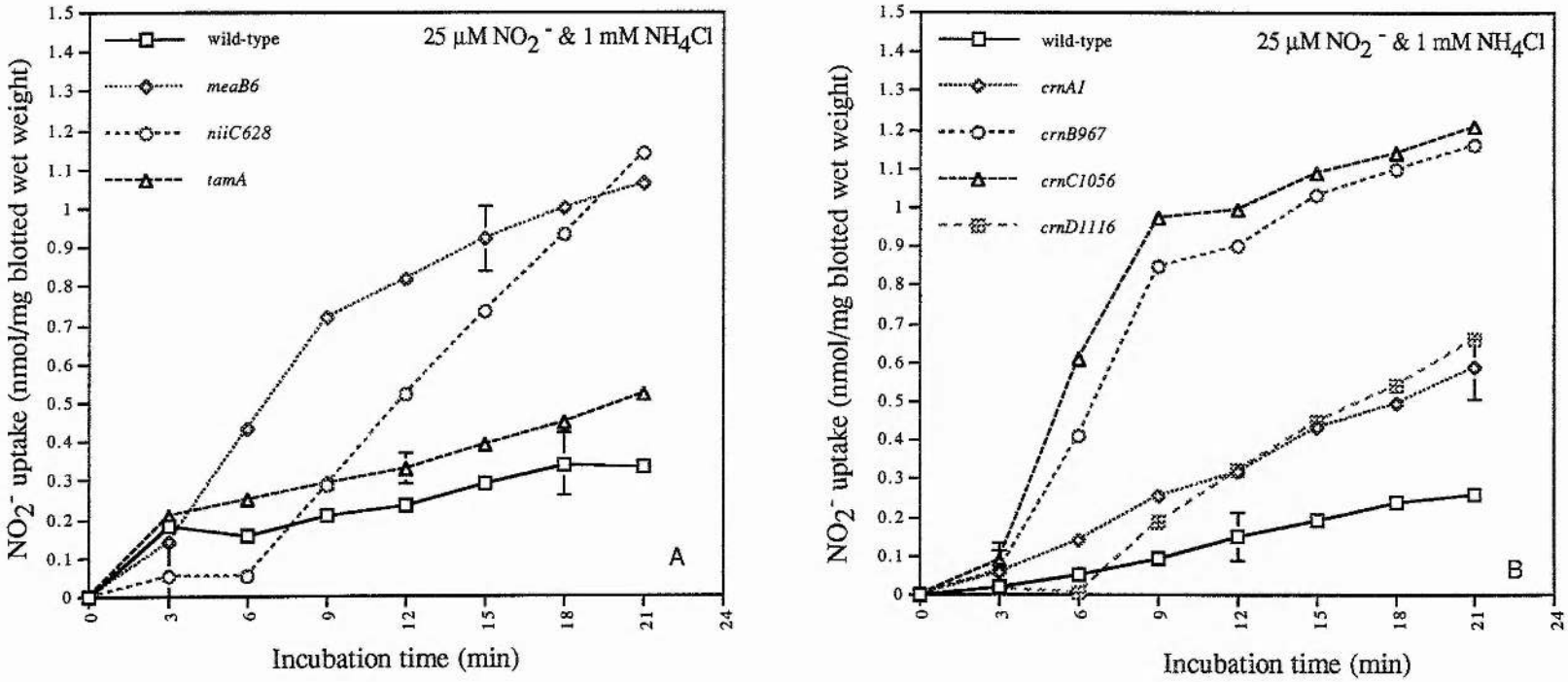


Figure 5.7. Inhibition Of Nitrite (25 µM) Uptake Activity By Ammonium In Older Cells. The effect of ammonium on the activity of the nitrate induced nitrite uptake system in older wild-type and various mutant cells. Mycelia were grown for16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with sodium nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (25 µM) and ammonium chloride (1 mM). Uptake values were measured at the indicated time, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than wild-type, ± 0.08; *meaB6*, ± 0083; *niiC628*, ± 0.087; *tamA105* ± 0.04; in **panel B** the maximum standard deviation was no greater than: wild-type, ± 0.063; *crnA1*, ± 0.079; *crnB967*, ± 0.054; *crnC1056*, ± 0.0.048; *crnD1116*, ± 0.025.

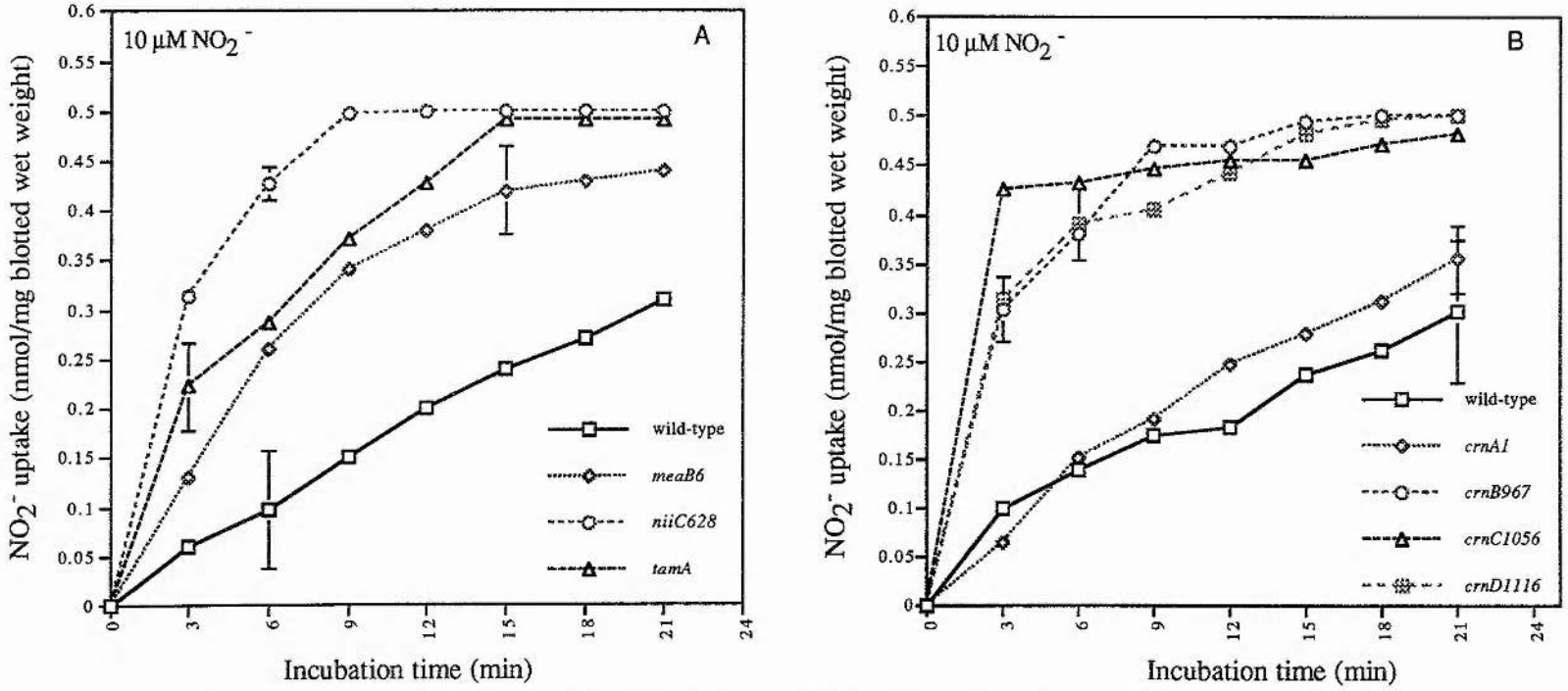


Figure 5.8. Uptake Activity Of Nitrite (10 µM) In Older Cells. The activity of the nitrate induced nitrite uptake system in wild-type and various mutant cells. Mycelia were grown for 16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with sodium nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (10 µM). uptake values were measured at the indicated times, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than: wild-type, ± 0.06; *meaB6*, ± 0.05; *niiC628*, ± 0.02; *tamA105*, ± 0.05. In **Panel B** the maximum standard deviation was no greater than: with-type, ± 0.073; *crnA1*, ± 0.035; *crnB967*, ± 0.034; *crnC1056*, ± 0.007; *crnD1116*, ± 0.37.

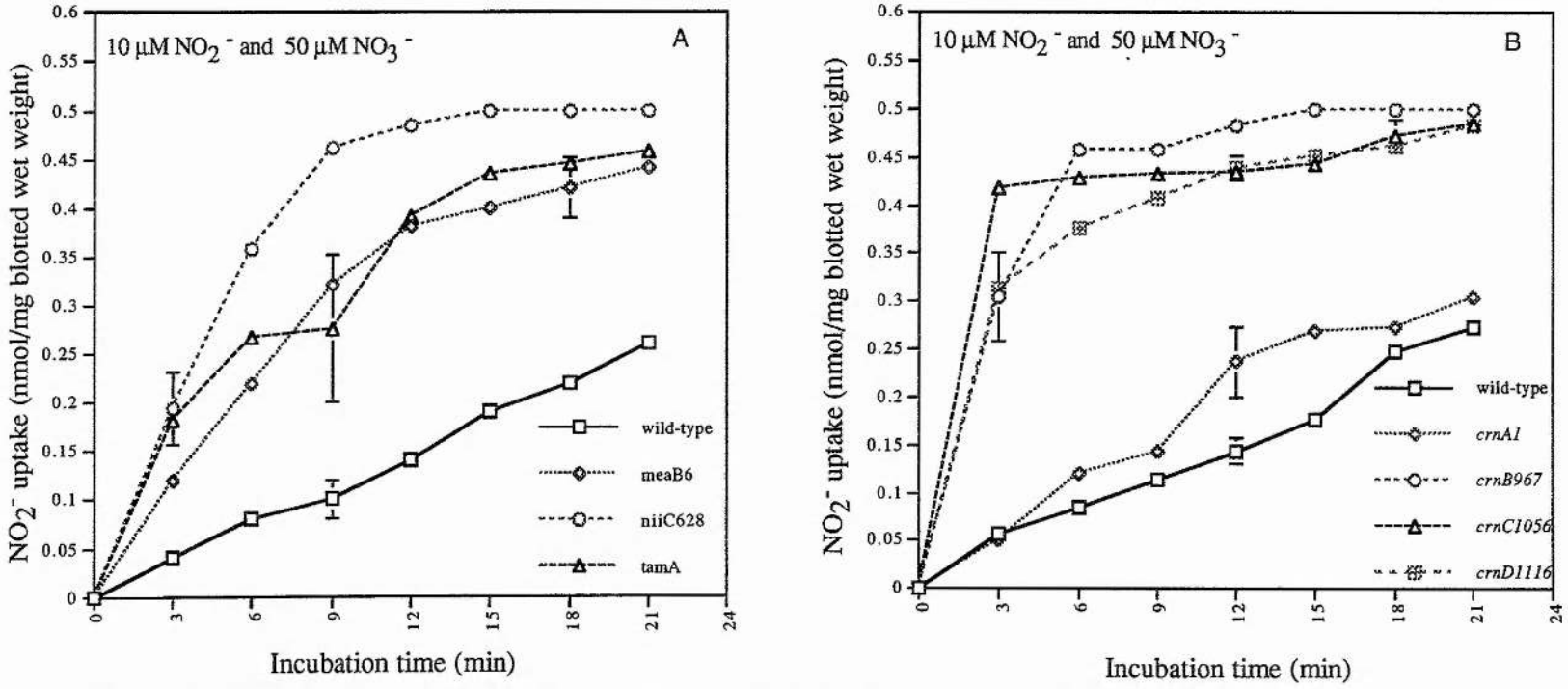


Figure 5.9. Inhibition Of Nitrite (10 µM) Uptake Activity By Nitrate In Older Cells. The effect of nitrate on the activity of the nitrate induced nitrite uptake system in wild-type and various mutant cells. Mycelia were grown for 16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with sodium nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (10 µM) and sodium nitrate (50 µM). uptake values were measured at the indicated times, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than: wild-type, ± 0.02; *meaB6*, ± 0031; *niiC628*, ± 0.037; *tamA105*, ± 0.075. In **Panel B** the maximum standard deviation was no greater than: wild-type, ± 0.013; *crnA1*, ± 0.037; *crnB967*, ± 0.046; *crnC1056*, ± 0.017; *crnD1116*, ± 0.013.

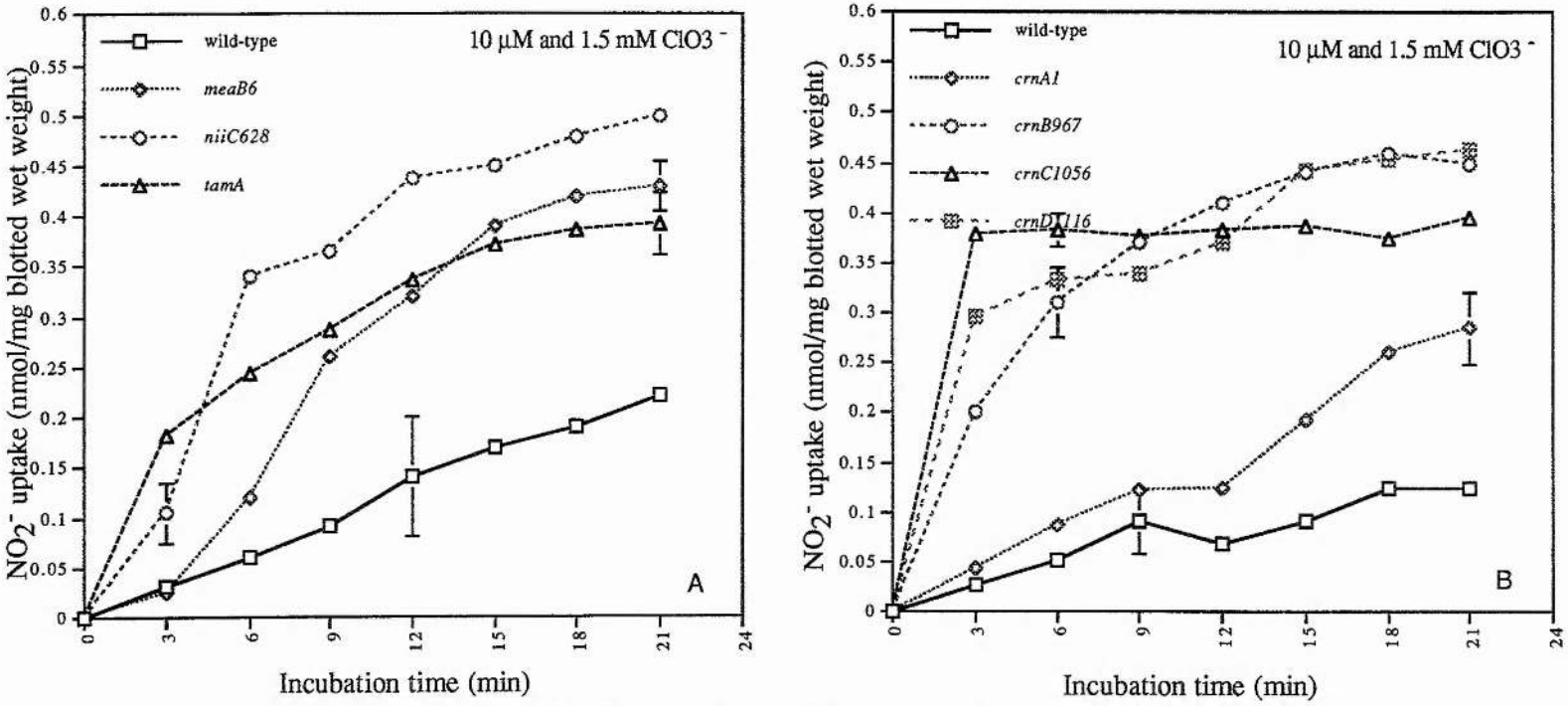


Figure 5.10. Inhibition Of Nitrite (10 µM) Uptake Activity By Chlorate In Older Cells. The effect of chlorate on the activity of the nitrate induced nitrite uptake system in wild-type and various mutants. Mycelia were grown for 16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with sodium nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (10 µM) and potassium chlorate (1.5 mM). Uptake values were measured at the indicated times, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than: wild-type, ± 0.06; *meaB6*, ± 0025; *niiC628*, ± 0.03; *tamA105*, ± 0.031. In **Panel B** the maximum standard deviation was no greater than: wild-type, ± 0.032; *crnA1*, ± 0.037; *crnB967*, ± 0.035; *crnC1056*, ± 0.017; *crnD1116*, ± 0.007.

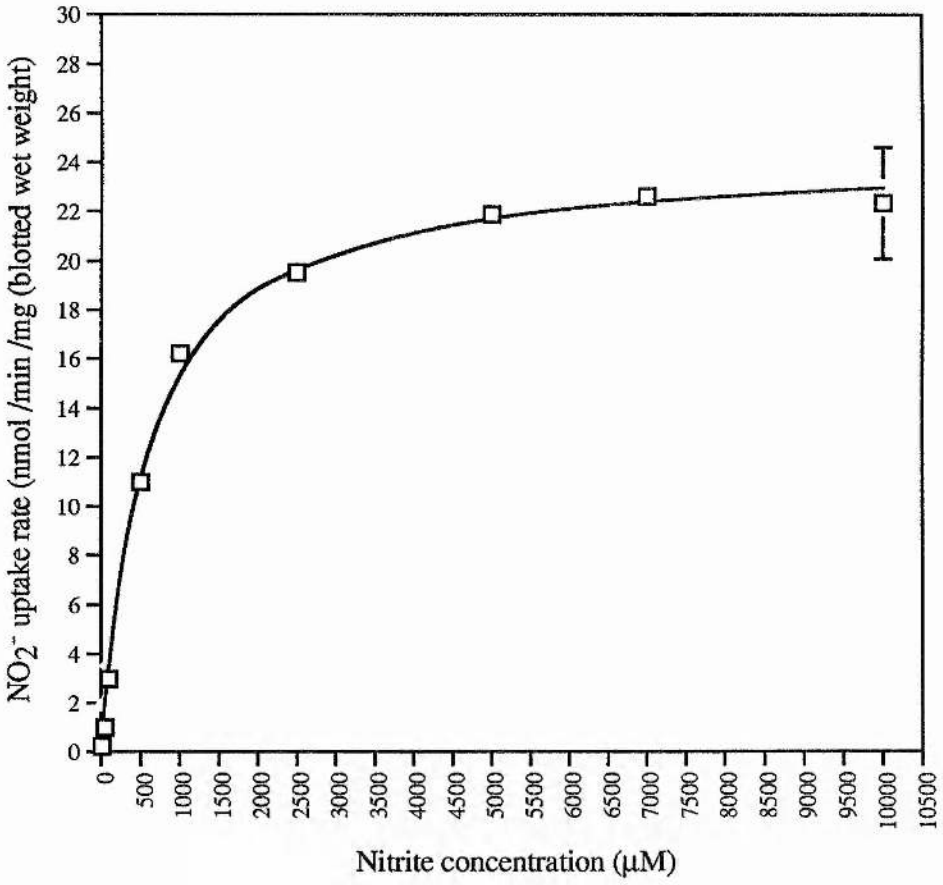


Figure 5.11. Graphical Representation Of Michaelis-Menten Equation For *meaB6*. Kinetic analysis of nitrite uptake by *meaB6* mutant grown for 16 h at 37°C in glucose minimal medium supplemented with urea (5 mM). Mycelia were induced by sodium nitrate (10 mM) 3 h before harvesting. Nitrate-induced cells were transferred to nitrite assay medium for nitrite uptake assay. Initial rate of nitrite uptake at different nitrite concentrations (10 μM; 50 μM; 100 μM; 500 μM; 1000 μM; 2500 μM; 5000 μM; 7000 μM; 10000 μM) in the medium was estimated at pH 6.5 in 1 mg/ml as the mean velocity corresponding to the mean substrate concentration in the same period. Values are the means ± standard deviation (SD) of three independent grow up experiments. The maximum standard deviation for any given point is detailed on the graph and was no greater than ± 2.26. This graphical representation has been drawn using CA-cricket graph III programme designed for the macintosh computers, while the Vmax and the Km values were obtained by using the programme ENZ FITTER designed for the IBM computers.

Table 5.1. Kinetic Parameters Of Nitrite Transport In *A. nidulans*.

Strain	Nitrite	
	K_m	V_{max}
wild-type	1004.00 ± 116.30	20.06 ± 0.60
crnA1	1170.57 ± 59.31	34.53 ± 0.46
crnB967	1035.68 ± 41.26	30.15 ± 0.31
crnC1056	882.87 ± 13.88	23.52 ± 0.09
crnD1116	972.81 ± 72.24	23.20 ± 0.43
meaB6	588.80 ± 48.24	24.26 ± 0.42
niiC628	688.00 ± 63.30	25.52 ± 0.53
tamA105	770.79 ± 32.33	22.22 ± 0.22

The results presented were from nitrate-induced cells. The Vmax value is in nmol. min^{-1}. mg^{-1} blotted wet weight and Km values are expressed in µM. Kinetic parameters were determined using the programme ENZ FITTER designed for the IBM computer.

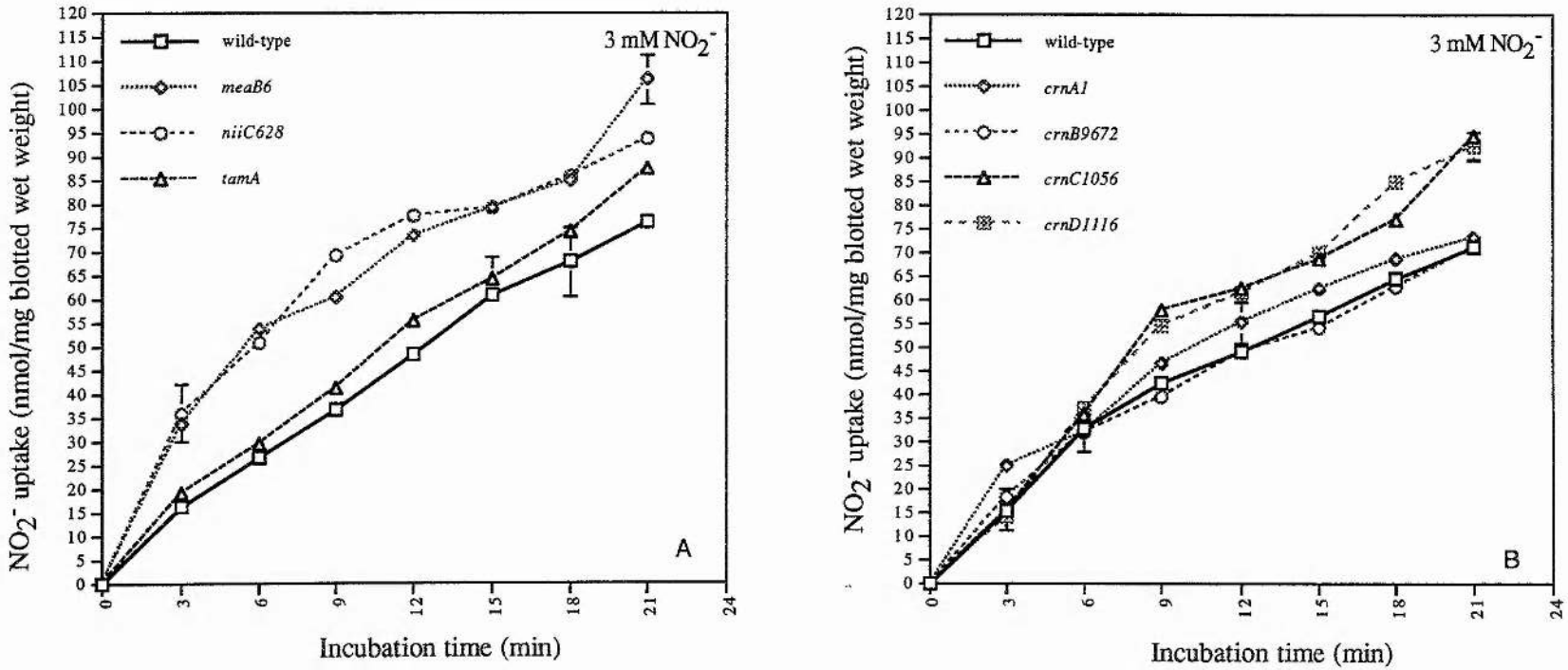


Figure 5.12 Uptake Activity Of Nitrite (3 mM) In Older Cells. The activity of the nitrate induced nitrite uptake system in the wild type and various mutants as indicated above. Mycelia were grown for16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with sodium nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (3 mM). Uptake values were measured at the indicated time, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than: wild-type, ± 7.2; *meaB6*, ± 6.1; *niiC628*, ± 5.2; *tamA105*, ± 4.5. In **Panel B** the maximum standard deviation was no greater than: wild-type, ± 4.9; *crnA1*, ±4.3; *crnB967*, ± 3.9; *crnC1056*, ± 4.4; *crnD1116*, ± 3.1.

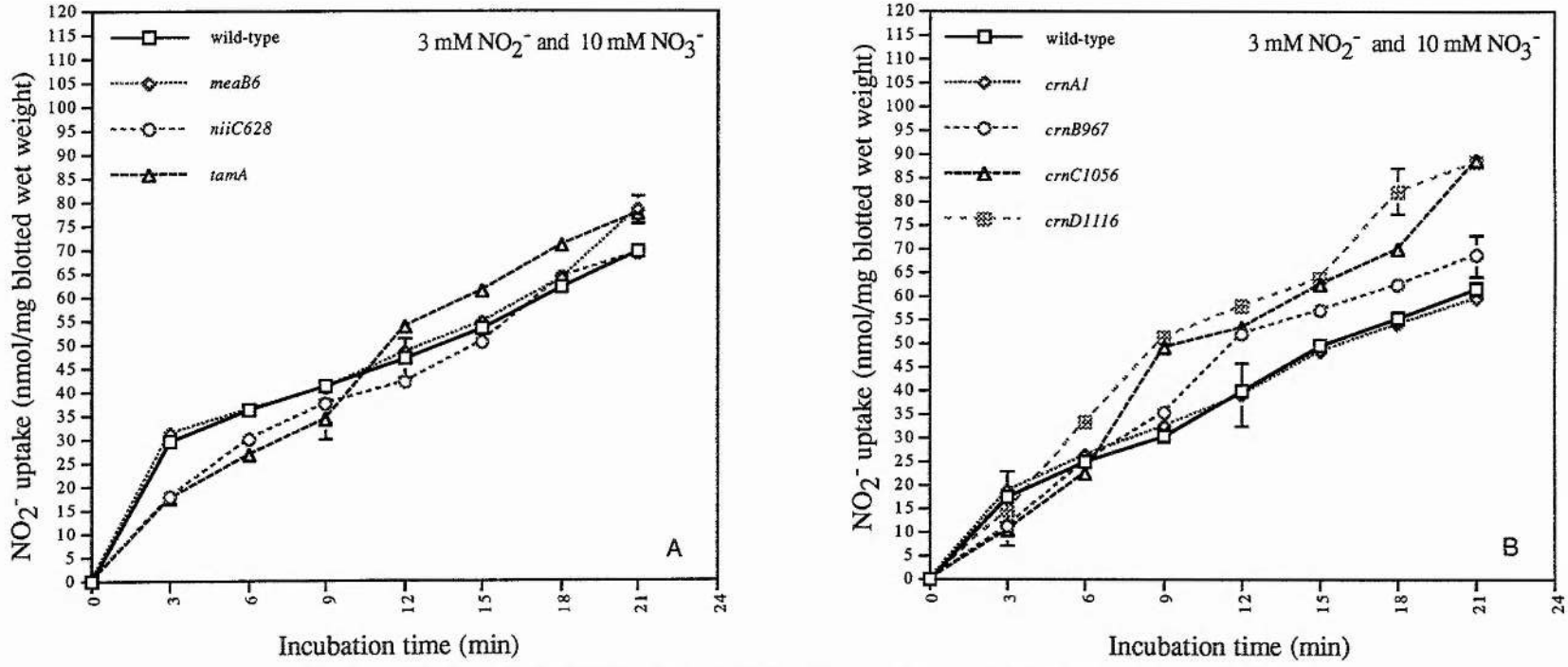


Figure 5.13. Inhibition Of Nitrite (3 mM) Uptake System By Nitrate In Older Cells. The effect of nitrate on the activity of the nitrate induced nitrite uptake system in wild-type and various mutant cells. Mycelia were grown for 16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with sodium nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (3 mM) and sodium nitrate (10 mM). Uptake values were measured at the indicated time, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than: wild-type, ± 4.2; *meaB6*, ± 3.0; *niiC628*, ± 4.1; *tamA105*, ± 4.2. In **Panel B** the maximum standard deviation was no greater than: wild-type, ± 5.5; *crnA1*, ± 6.6; *crnB967*, ± 4.5; *crnC1056*, ± 3.4; *crnD1116*, ± 4.8.

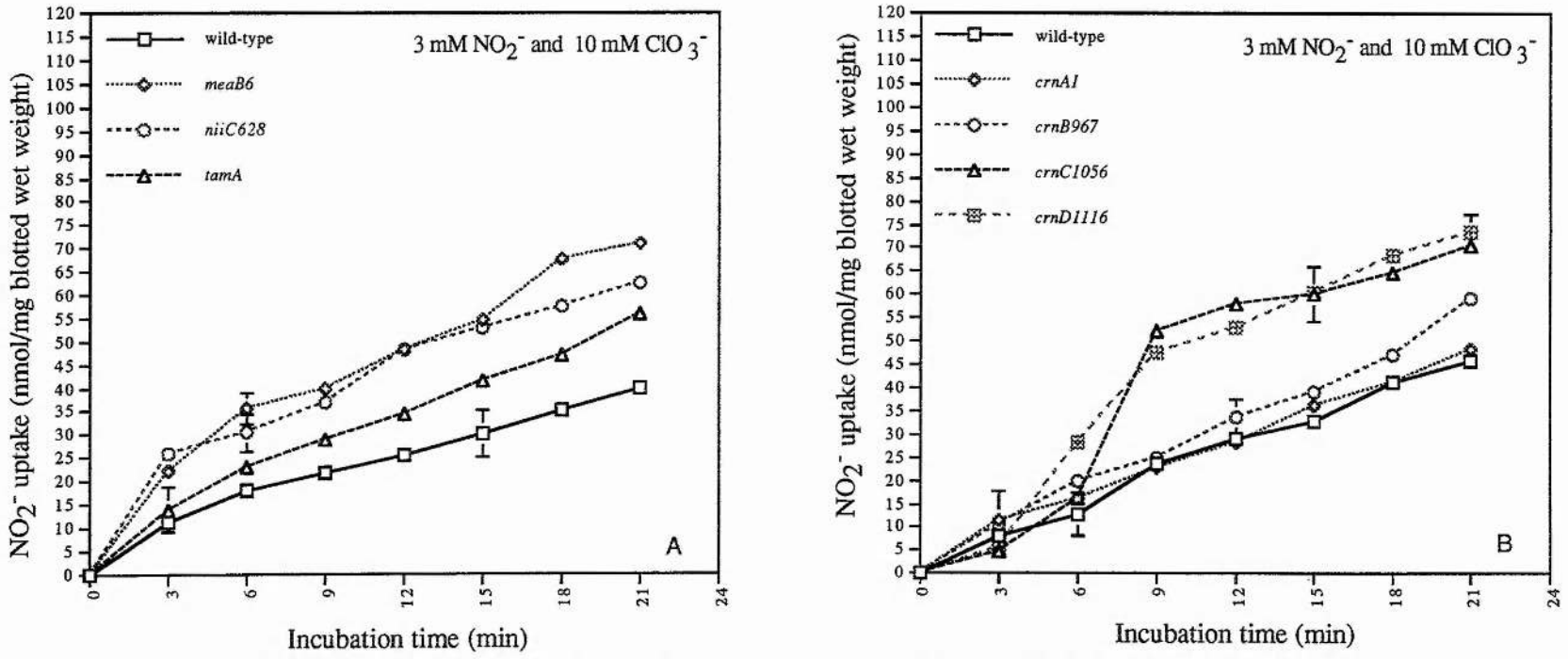


Figure 5.14. Inhibition Of Nitrite (3 mM) Uptake Activity By Chlorate In Older Cells. The effect of chlorate on the activity of the nitrate induced nitrite uptake system in older wild-type and various mutant cells. Mycelia were grown for 16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with sodium nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (3 mM) and potassium chlorate (10 mM). Uptake values were measured at the indicated time, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than: wild-type, ± 6.5; *meaB6*, ± 3.4; *niiC628*, ± 4; *tamA105*, ± 4.9. In **Panel B** the maximum standard deviation was no greater than: wild-type, ± 4.6; *crnA1*, ± 6.4; *crnB967*, ± 3.9; *crnC1056*, ± 6.0; *crnD1116*, ± 3.8.

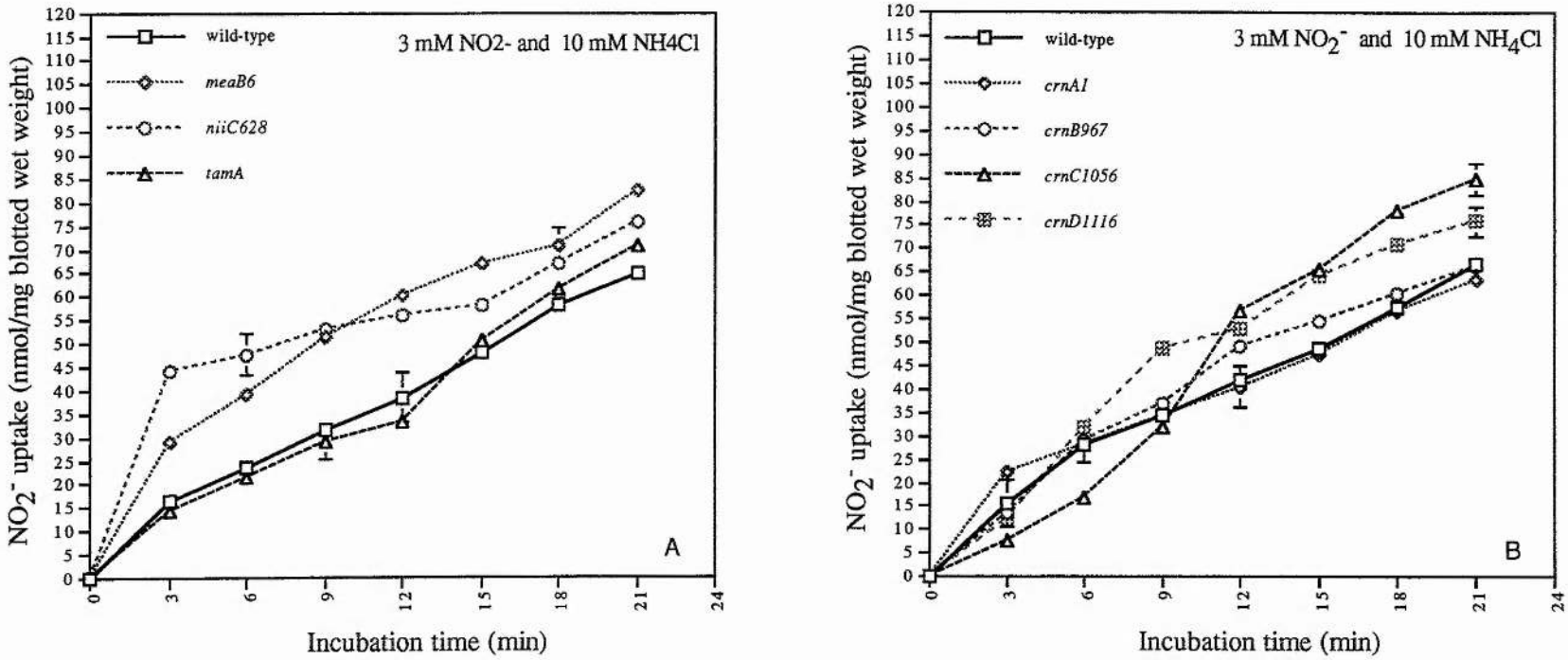


Figure 5.15. Inhibition Of Nitrite (3 mM) Uptake Activity By Ammonium In Older Cells. The effect of ammonium on the activity of the nitrate induced nitrite uptake system in older wild-type and various mutant cells. Mycelia were grown for 16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were washed (by nitrogen-free media incubated at the same temperature as the cells 1 h before) and transferred to a new media with sodium nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Treated cells were washed as before and transferred to nitrite assay medium containing sodium nitrite (3 mM) and ammonium chloride (10 mM). Uptake values were measured at the indicated time, and are the means ± standard deviation (SD) of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation in **Panel A** was no greater than wild type, ± 5.3; *meaB6*, ± 3.9; *niiC628*, ± 4.3; *tamA105*, ±3.8. In **Panel B** the maximum standard deviation was no greater than: wild type, ± 4.9; *crnA1*, ± 4.3; *crnB967*, ± 4.4; *crnC1056*, ± 3.4; *crnD1116*, ± 3.2.

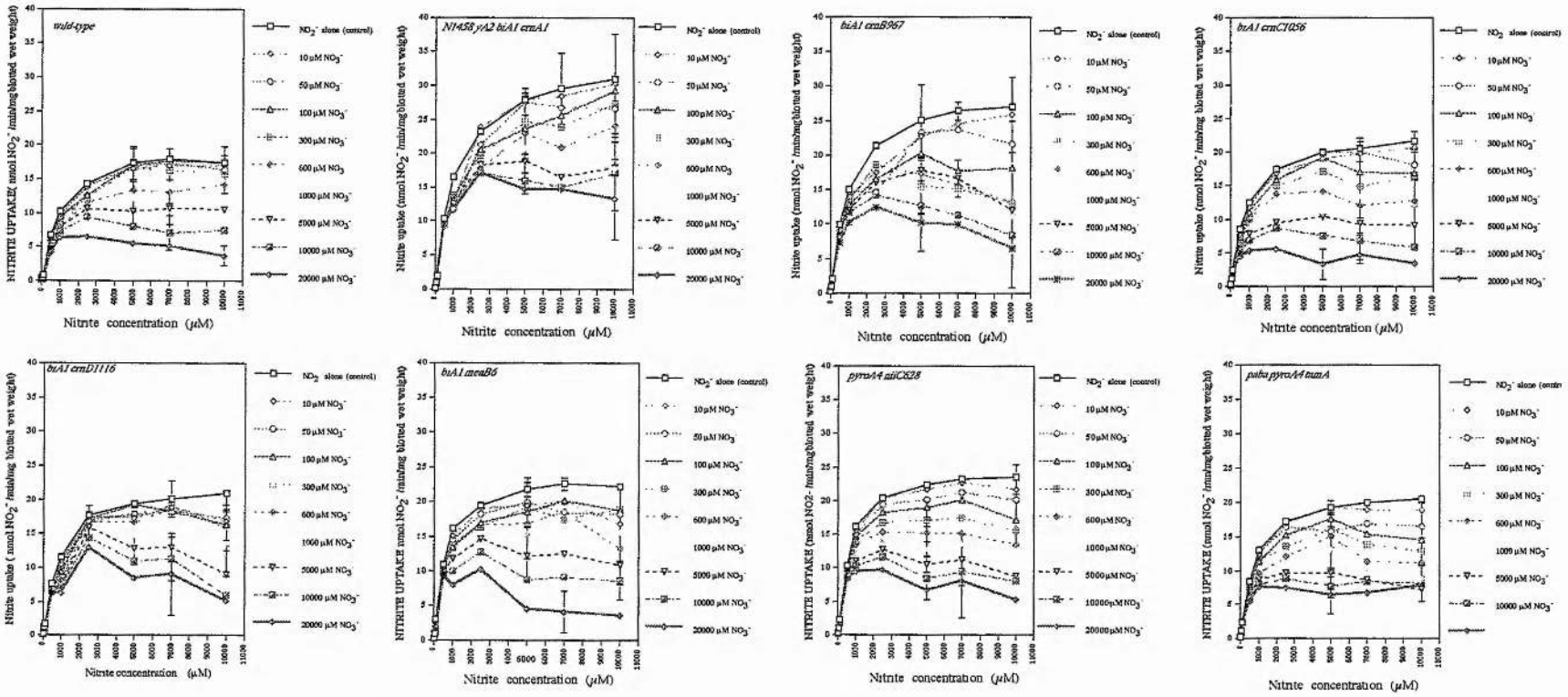


Figure 5.16. Inhibition Of Nitrite (10, 50, 100, 500, 1000, 2500, 5000, 7000, 10000 μM) Uptake By Different Nitrate Concentrations In Older Cells. The effect of various nitrate concentrations on the activity of the nitrate induced nitrite uptake system in the wild-type and various mutant cells. Mycelia weregrown for 16 h at 37°C in glucose minimal medium supplemented with urea (5 mM) as the sole nitrogen source. Mycelial cells were treated with nitrate (10 mM) as sole nitrogen source, 3 h before harvesting. Initial uptake rates (see materials and methods)were determined at the nitrite concentrations indicated above in the absence or in the presence of nitrate (10 μM; 50 μM; 100 μM; 300 μM; 600 μM; 1000 μM; 5000 μM; 10000 μM; and 20000 μM). Values are the means± standard deviation of three independent grow up experiments. The point which showed the highest standard deviation is specified for each graph, where the maximum standard deviation was no greater than: wild-type, ± 4.5; *crnA1*; ± 5.97: *crnB967*; ± 6.96;*crnC1056*; ± 7.24;*crnD1116*, ± 6.03;*meaB6*, ± 5.37;*niiC628*, ± 5.56;*tamA105*, ± 5.49.

CHAPTER SIX

CHARACTERISATION OF THE *cnx* MUTANTS.

6.1 The Objectives Of This Research Section.

The first aim of this part of work was to isolate and sequence molybdenum cofactor genes. The second objective was to generate chlorate resistant *cnx* mutants either temperature-conditional or non-conditional, to provide mutants that could be sequenced in parallel with the wild-type allele, in order to determine the sequence changes in these mutants and the inferred protein defects. Such combined molecular and biochemical studies on these mutants should provide basic information on their role in molybdenum cofactor biosynthesis.

In other words, this study should allow comparisons at the genetical, molecular and biochemical levels with already isolated and sequenced equivalent genes from other organisms or even unearth new genes to study.

6.2 Genetic Analysis.

6.2.1 Isolation Conditions Of *cnx* Mutants Which Have Been Genetically Analysed.

The confirmed chlorate resistant *cnx* mutants were isolated according to growth criteria listed in Table 3.1 and discussed in Chapter three. After spontaneous, NTG, or DEO mutagenesis of

either wild-type or a genetically engineered *gdh-niaD* constructed transformant, 2082 *cnx* mutants (including 20 temperature-conditional mutants) were generated from a total of 11,807 confirmed chlorate resistant mutants (about 18% are *cnx* mutants). Within 2082 *cnx* mutants phenotypically recognised, 113 were isolated after spontaneous mutagenesis, 375 after NTG and 1594 were isolated after DEO mutagenesis. Of the 2082 isolated *cnx* mutants, 456, including the temperature-conditional strains, were genetically analysed by functional complementation in heterokaryons. Heterokaryon functional complementation tests were carried out by point inoculum on minimal medium with 10 mM nitrate using representative alleles of the previously isolated non-conditional *cnx* mutant (Cove and Pateman, 1963; MacDonald and Cove, 1974) *cnxA5*, *cnx B11*, *cnxC3*, *cnxE3*, *cnxF7*, *cnxG4* and *cnxH4* were used in this complementation study. The details and isolation conditions for each particular *cnx* mutant are shown in Table 6.1. These data indicate that 39 of the 456 analysed *cnx* mutants (approx 9%) are alleles of *cnxA*, 130 (approx 30%) are alleles of *cnxB*, 43 (approx 10%) are alleles of *cnxC*, 54 (approx 12%) are alleles of *cnxE*, 100 (approx 22%) are alleles of *cnxF*, 68 (approx 15%) are alleles of *cnxG* and 22 mutants (approx 5%) are alleles of *cnxH*. Altogether, 20 were found to be temperature-conditional mutants consist of 10 thermo-sensitives and 10 cryso-sensitives (i.e. cold-sensitive).

Isolation Conditions Of *cnxA* Mutants.

The results presented in Table 6.1 indicate that glutamate was not a nitrogen source of choice for generating *cnx* mutants when wild-type strains were mutagenised by either spontaneous or DEO mutagenesis. However, 25% of the genetically analysed *cnx* mutants were found to be alleles of *cnxA* when glutamate was used at 25°C as the sole nitrogen source to grow wild-type treated with the NTG mutagen. No *cnxA* mutants were obtained at 37°C under the same mutagenic conditions. Additionally, *no cnxA* alleles were generated at 37°C after DEO treatment of the genetically engineered *gdh-niaD* transformants SAA1040, SAA1032 or SAA1023b (see section 2.1 for a description of these strains) when proline, ammonium, or urea was used as the sole nitrogen sources.

Isolation Conditions Of *cnxB* Mutants.

The results presented in Table 6.1 indicate that after NTG mutagenesis of wild-type strains at 37°C with glutamate used as the sole nitrogen source, 60% of the analysed mutants were found to be alleles of *cnxB*. After DEO treatment of the *gdh-niaD* transformant SAA1040 generated at 37°C, approximately 50% *cnxB* mutants when either ammonium or urea were used as sole nitrogen sources. However, 38% *cnxB* mutants were generated when ammonium was used with the strain SAA1032 after DEO mutagenesis. In contrast, *no cnxB* alleles were generated when urea was used with this latter strain, under the same mutagenic conditions.

Isolation Conditions Of *cnxC* Mutants.

Table 6.1 results indicate that when uric acid was used in plates incubated at 37°C, after DEO mutagenesis of the wild-type strain, approximately 95% of the analysed mutants were alleles of *cnxC* . 0% to 3% *cnxC* alleles were obtained at 25°C, using the same mutagenic regime. However, no *cnxC* mutants were isolated at 25°C when uric acid was used with wild-type strains treated with NTG. Additionally, no single *cnxC* mutant allele was obtained with any of the *gdh-niaD* transformants after DEO mutagenesis with any nitrogen source.

Isolation Conditions Of *cnxE* Mutants.

When uric acid was used at 25°C with wild-type strains and after NTG treatment, only 3% of the analysed mutants were alleles in *cnxE*, whilst, no *cnxE* alleles were obtained at 37°C. Surprisingly, these alleles formed approximately 50% to 70% of the total analysed mutants when either proline or urea were used at 37°C with *gdhA-niaD* transformants SAA1040 or SAA1032 strains after DEO treatment.

Isolation Conditions Of *cnxF* Mutants.

The frequency of *cnxF* alleles ranged between 30% and 50% with all *gdh-niaD* transformants treated with DEO at 37°C. Such high frequencies were also obtained at 25°C but not 37°C after either NTG or spontaneous mutagenesis of wild-type strains, especially when either uric acid, glutamate or proline were used as the sole

nitrogen source. However, no *cnxF* mutant alleles were obtained at 37°C when ammonium was used as the nitrogen source to grow wild-type cells treated with the NTG mutagen.

Isolation Conditions Of *cnxG* Mutants.

When wild-type strains were mutagenised with NTG, on uric acid medium at 25°C, 35% *cnxG* mutant alleles were synthesised. In addition, 38% alleles were obtained with ammonium at 37°C when the SAA1032 strain was treated with DEO. Unexpectedly, no *cnxG* alleles were generated when either SAA1040 or SAA1032 transformants treated with DEO, in particular when either proline or urea were used as sole nitrogen sources.

Isolation Conditions Of *cnxH* Mutants.

It is clear from the data presented in Table 6.1 that *cnxH* alleles formed the lowest frequency of *cnx* mutant alleles isolated. Such low frequency (i.e. approx 5%) was obtained with either wild-type strains treated with NTG or with transformants SAA1040, SAA1023b treated with DEO. *cnxH* mutant alleles of the wild-type origin were obtained when either glutamate, proline, or uric acid were used at both selection temperatures. The mutant alleles that came from SAA1040 were obtained when urea was the sole nitrogen source, whilst, the mutants from transformant SAA1023b were generated when either proline or ammonium was used. However, no *cnxH* mutant alleles were isolated when uric acid or glutamate were used with wild-type treated by DEO mutagen and selected at

37°C. Additionally, *cnxH* mutant alleles were not obtained also from either SAA1040 or SAA1032 after DEO mutagenesis or when proline, urea or ammonium were used as sole nitrogen sources.

6.3 Characterisation Of The Temperature-Conditional *cnx* Mutants.

Originally for selection purposes two series of growth test plates (see section 3.3) were inoculated at the same time, one set was incubated at 25°C and the other at 37°C. Certain *cnx* mutants showed growth with either nitrate or adenine at the permissive temperature but not at the non-permissive temperature. Such mutants were considered to be temperature-conditional cnx strains and were classified as thermo- and cryso-sensitive mutants accordingly (Table 4.1). The thermo-sensitive *cnx* mutants are defined as chlorate resistant mutants which were selected at 37°C and showed growth on either nitrate or adenine at 25°C but not 37°C. However, some of these mutants showed growth at 25°C on nitrate but not adenine and considered as thermo-sensitive mutants to nitrate but totally mutants on adenine as sole nitrogen source. All cryso-sensitive mutants which were selected on chlorate at 25°C showed growth on either nitrate or adenine at 37°C but not 25°C.

Within the 2082 selected *cnx* mutants, 20 were temperature-conditional. Of these 10 were thermo-sensitive and 10 found to be cryso-sensitive (or cold-sensitive). Of the 10 thermo-sensitive mutants only one was allele of the *cnxA*, one was allele of the *cnxB*,

two of the *cnxC*, one of the *cnxE* two of the *cnxF* and three of the *cnxH*. Detailed information concerned with these mutants are presented in Table 6.2.

Five thermo-sensitive mutants (i.e. *cnxB224*, *cnxF384*, *cnxH251*, *cnxH255* and *cnxH261*) were isolated when urea was used with the transformant strain SAA1040 and after DEO mutagenesis. The other 5 mutants (i.e. *cnxA140*, *cnxC232*, *cnxC465*, *cnxE246* and *cnxF142*), were generated also after DEO mutagenesis of transformant strain SAA1023b and when proline was used as the sole nitrogen source. However, all the cryso-sensitive mutants were derived from wild-type strains. Four of these were alleles of *cnxB*, three were alleles of *cnxC* and three were alleles of *cnxF*. Within the four *cnxB* mutants three (i.e. *cnxB75*, *cnxB97* and *cnxB1236*) were generated with glutamate as sole nitrogen source and after NTG mutagenesis, the fourth mutant (i.e. *cnxB1277*) was generated with uric acid as the nitrogen source using the same mutagen. However, all the *cnxC* mutants (i.e. *cnxC418*, *cnxC485*, *cnxC399*) were obtained with proline as sole nitrogen source after NTG mutagenesis. In addition, two of the *cnxF* (i.e. *cnxF910* and *cnxF822*) mutants were isolated after spontaneous mutagenesis, whilst the third mutant (i.e. *cnxF482*) was obtained after NTG and all of the *cnxF* mutants proline was the sole nitrogen source detailed results are shown in Table 6.2. Additionally, a summary of the total number of genetically analysed *cnx* mutations in each locus is presented in Table 6.3.

The results presented in Figure 6.1 shows the growth patterns of certain temperature-conditional *cnx* mutants at three different temperatures (i.e. 25°C, 37°C and 42°C). Such growth patterns shown in figure 6.1 indicate that both types of representative mutants i.e. thermo-sensitives (*cnxH255* and *cnxB224*) and the cryso-sensitives (*cnxB97* and *cnxF822*) showed high resistance to chlorate (300 mM) and wild-type levels of growth on ammonium (10 mM) as the sole nitrogen source at three different temperatures. The growth patterns of these mutants on either nitrate (10 mM) or adenine (5 mM) as the sole nitrogen source is shown in Figure 6.2. Both thermo-sensitive mutants were unable to grow at 37°C or 42°C on either nitrate or adenine as sole nitrogen sources. However, at 25°C the mutant *cnxH255* showed wild-type level of growth on either nitrate or adenine, whereas, *cnxB224* was unable to grow on adenine at the same temperature. Furthermore, both cryso-sensitive mutants at 25°C showed a complete mutant phenotype (i.e. did not show any growth) on either nitrate or adenine whilst, both showed wild-type levels of growth at 37°C and 42°C on either nitrate or adenine as sole nitrogen source.

6.4 Comparison Of Temperature-Conditional Mutant Frequencies With Previous Studies.

These results have been compared with Cove's original results (MacDonald and Cove, 1974). The data in Table 6.4 indicates that the frequency of temperature-sensitive mututations in the *cnxG* gene is much lower than that in the other genes especially *cnxH*.

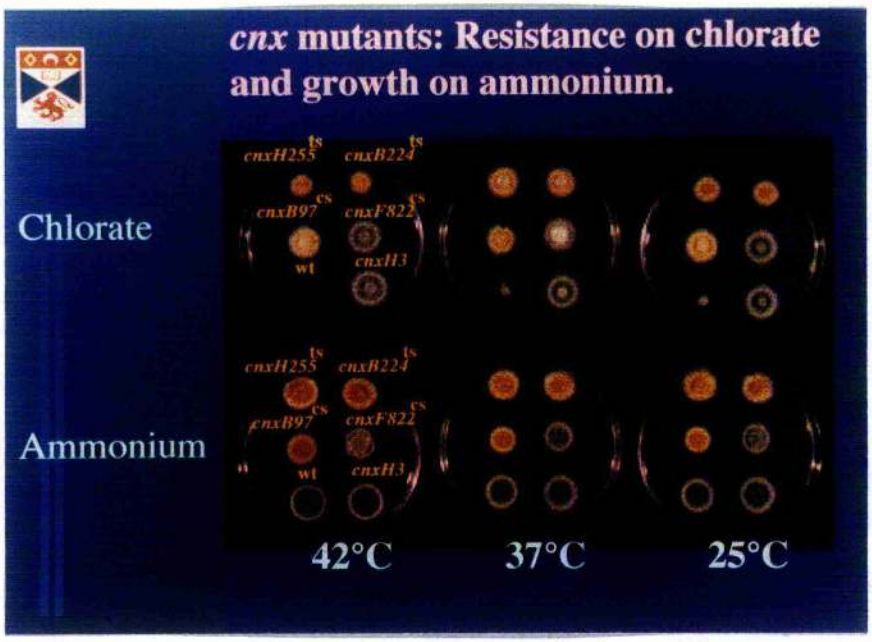


Figure 6.1. Chlorate Resistance And Growth Patterns Of Certain Temperature-Conditional *cnx* Mutants.

Several *cnx* mutants were growth tested (and shown here) at three different temperatures (i.e. 25°C, 37°C and 42°C), on glucose minimal medium, pH 6.5 containing potassium chlorate (300 mM) and proline (10 mM) as the sole nitrogen source. In addition, mutants were tested for growth on ammonium (10 mM) as the sole nitrogen source. All mutants showed high resistance to chlorate and wild-type levels of growth on ammonium as the sole nitrogen source. symbol ts: denotes thermo-sensitive, symbol cs: denotes cryso-sensitive.

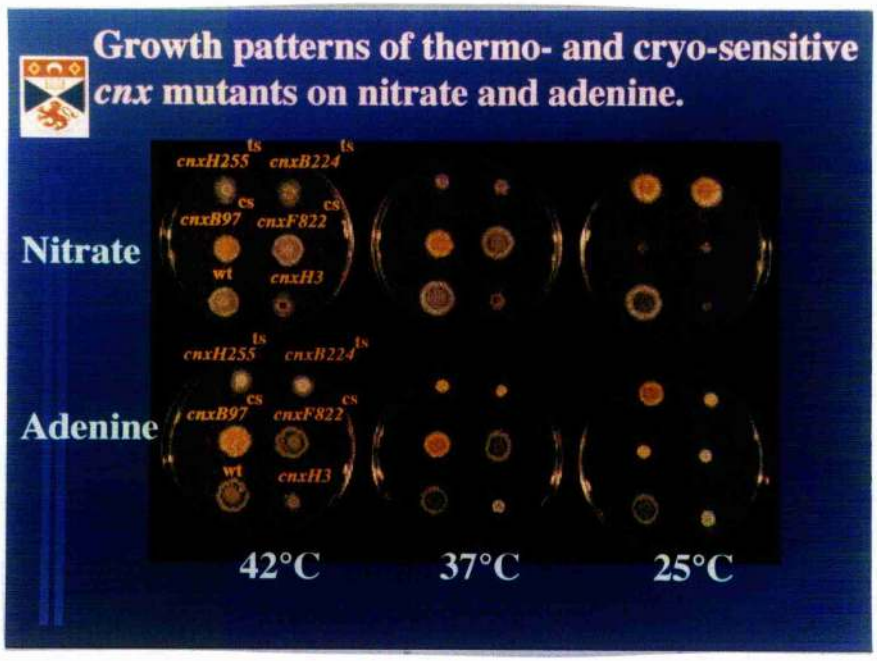


Figure 6.2. Growth Patterns Of Temperature-Conditional *cnx* Mutants On Nitrate And Adenine.

several mutants were tested (and shown here) at three different temperatures (i.e. 25°C, 37°C and 42°C) on glucose minimal medium pH 6.5, containing either nitrate (10 mM) or adenine (5 mM) as the sole nitrogen source. Both thermo-sensitive mutants (i.e. *cnxH255* and *cnxB224*) did not show growth on nitrate at either 37°C or 42°C. However, at 25°C the *cnxH255* mutant showed wild-type levels of growth on either nitrate or adenine, whereas, strain *cnxB224* was totally mutant when grown on adenine at the same temperature. Both cryso-sensitive mutants (i.e. *cnxB97* and *cnxF822*) showed growth at 25°C on either nitrate or adenine, whilst both mutants showed wild-type levels of growth at either 37°C or 42°C with either nitrate or adenine. symbol ts: denotes thermo-sensitive, symbol cs: denotes cryso-sensitive mutant.

Furthermore, genetic analysis of *cnx* mutants obtained in this study as well as in the previous studies (MacDonald and Cove, 1974) showed that mutations in *cnxH* locus occur much less frequently and only about 5% *cnxH* alleles were generated in either of the studies under the same isolation conditions. However, within this low frequency between 20% (i.e. previous studies) and 30% (i.e. this study) of the thermo-sensitive mutants were alleles of the *cnxH.* These results might be explained as follows, since these *cnxH* mutants were isolated in both studies after chemical mutagenesis (i.e. NTG or DEO) the low number of these mutants could arise as a result of this particular *cnx* gene being more resistant to mutagenic treatment than others. However, the selection of a relatively high percentage of thermo-sensitive *cnxH* mutants as compared to the total generated mutants of the same locus, could be related to a small change in the protein. This small change can be explained as follows. Since such generated mutants (i.e. thermo-sensitive mutants) were obtained after mutagenic treatments so it is possible that such mutagens might induce high frequency of multi-site mutations. That means, such mutations which cause small change in the protein might came as a result of at least a second induced mutation at a site other than the active site. The protein in the second site may not be essential for the activity of the enzyme, but it could be involved in the folding process of the essential protein. That means interruption in the folding process of the protein could change the configuration of the that essential protein and makes it inactive at the non-permissive

temperature but normal at the permissive one (for further discussion see discussion of NR activity). Additionally, some of the temperature-sensitive *cnxH* mutants isolated in this study and in Cove's study were found to be temperature-sensitive on nitrate, but unable to grow on adenine or hypoxanthine (see Table 6.4). This would suggest that the molybdoenzyme (i.e. PH) has a more stringent requirement for the integrity of the molybdenum cofactor than has NR enzyme.

6.5 Nitrate Reductase Activities In Thermo-Sensitive *cnx* Mutants.

The NR protein produced by certain thermo-sensitive *cnx* mutants isolated in this study were examined *in vitro* for temperature stability. Of the ten isolated thermo-sensitive mutants, eight were examined for the stability of the enzyme. One mutant was an allele at *cnxA* (i.e. *cnxA140*), two were alleles of the *cnxC* (i.e. *cnxC232* and *cnxC465*), two *cnxF* (i.e. *cnx142* and *cnxF384*) and three *cnxH* (i.e. *cnxH251*, *cnxH255* and *cnxH261*). None of the cryso-sensitive mutants were included in this study.

Strains were grown at 25°C on minimal medium with nitrate (10 mM). The crude extract was incubated at 35°C at a time course intervals of 3 min each starting from o min up to 21 min. At a time intervals of 40 sec the crude extract from each incubation period was transfered from ice to an assay mixture incubated at 25°C. The assay miture with the extract were incubated at 25°C for 20 min.

After stopping the reaction 1 ml of sulphanilamide and 1 ml of NED solution were added, mixed and incubated at room temperature for 20 min for colour development. The resultant colour was proportional to the amount of nitrite present and was estimated by determining the absorbance at 540 nm spectrophotometrically.

The results presented in Figure 6.3 indicated that the enzyme produced by *cnxA140* mutant was more heat liable than that of the wild-type, where the half-life of the mutant enzyme was considerably shorter (i.e. 9.3 min) than the wild-type enzyme (i.e. 17.5 min).

Both temperature-sensitive *cnxC* mutants possessed enzyme activity with a half-life not that much different from the wild-type between 11.4 min for *cnxC232* and 13.1 min for *cnxC465* as compared with 18.1 min wild-type enzyme (Figure 6.4). Similarly, Figure 6.5 shows that there was no significant difference between the half-life of the enzyme produced by *cnxF* mutants (i.e. *cnxF384*: 13.8 min and *cnxF142*: 15.2 min) and that produced by the wild-type strain (i.e. 18.3 min).

Finally, two thermo-sensitive *cnxH* mutants showed an enzyme activity with a half-life much shorter (i.e. 5.5 min in mutant *cnxH255*: and 5.2 min in mutant *cnxH261*:) than that of the wild-type, whereas, the mutant *cnxH251* showed enzyme activity with a half-life 12.4 min as compared to 17.5 min for the wild-type (Figure 6.6).

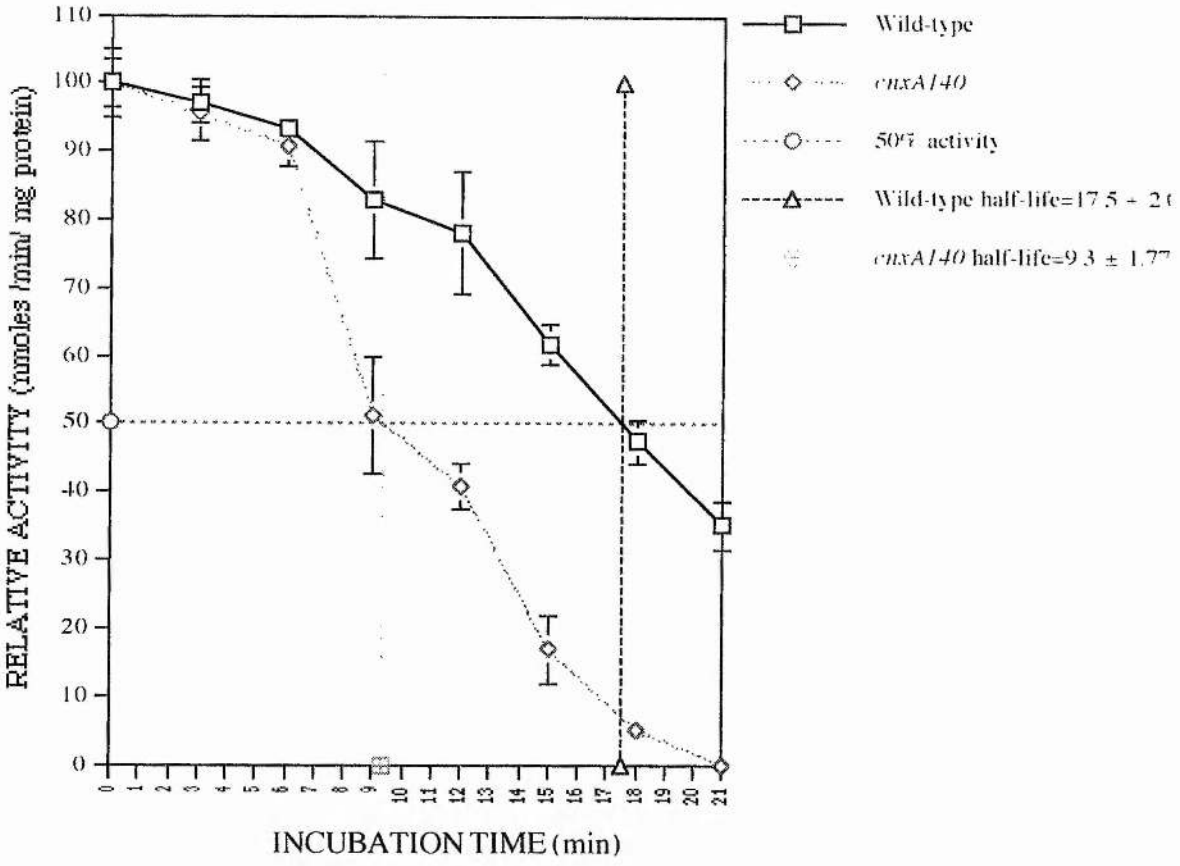


Figure 6.3. Relative Activities (Specific activities normalised to the maximum specific activity at time =0) And Half-Lives At 35°C Of Nitrate Reductase Enzyme In The Wild-type And The *cnxA* Temperature-Sensitive Mutant (*cnxA140* ^{ts}). The wild-type and the mutants were grown for 21 h at 250 rpm, at 25°C in glucose minimal medium (with supplements), at pH6.5 with sodium nitrate (10 mM). Cell-free extracts were assayed at 25°C for 20 min. Soluble protein in extracts was determined using crystalline bovine serum albumin as standard (Bio-Rad protein standard). Enzyme activities are given as nanomoles of NADPH oxidised per min per milligram of protein. Values are mean ± standard deviation (SD) of two independent grow up experiments. Wild-type specific activity is 62.79 ± 5.12. The *cnxA140* ^{ts} specific activity is 45.20 ± 3.56.

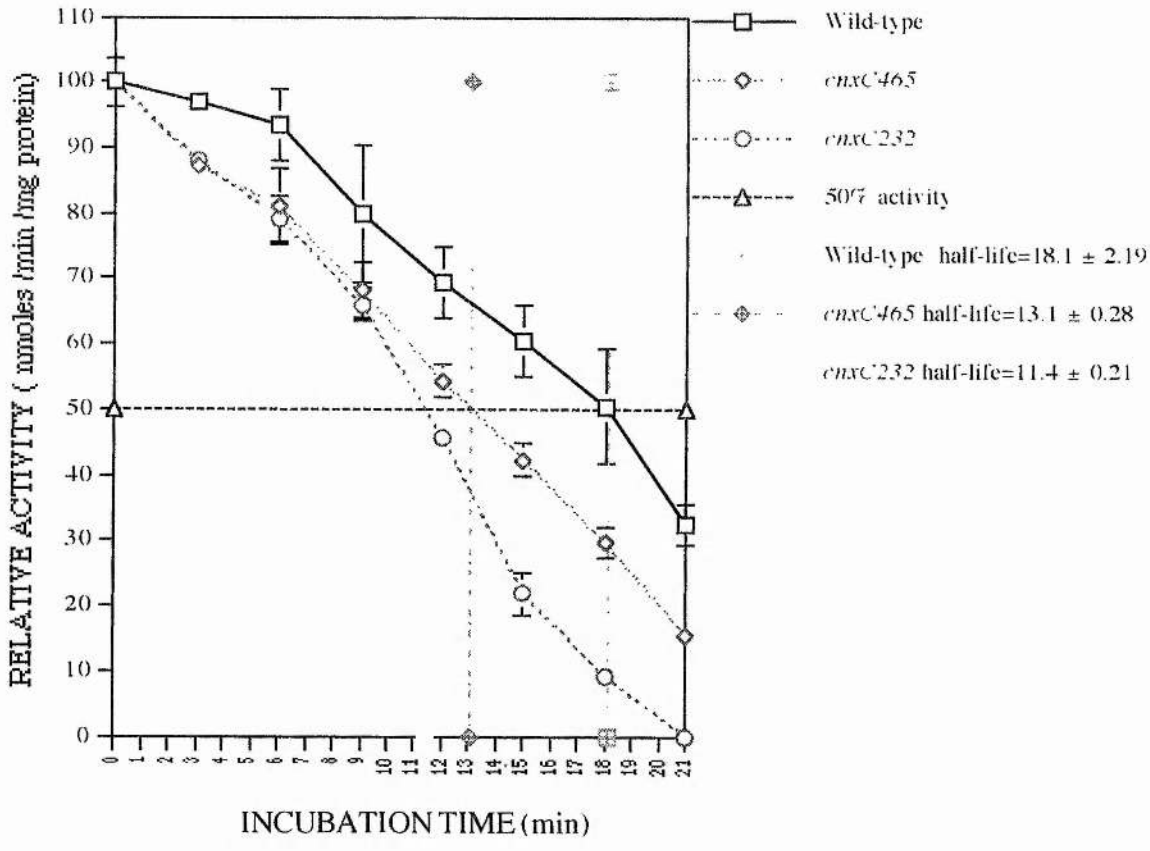


Figure 6.4. Relative Activities (specific activities normalised to the maximum specific activity at time =0) And Half-Lives At 35°C Of Nitrate Reductase Enzyme In The Wild-Type And Two *cnxC* Temperature-Sensitive Mutant (*cnxC232* ^{ts} and *cnxC465* ^{ts}). The wild-type and the mutants were grown for 21 h at 250 rpm, at 25°C in glucose minimal medium (with supplements), at pH6.5 with sodium nitrate (10 mM). Cell-free extracts were assayed at 25°C for 20 min. Soluble protein in extracts was determined using crystalline bovine serum albumin as standard (Bio-Rad protein standard). Enzyme activities are given as nanomoles of NADPH oxidised per min per milligram of protein. Values are mean ± standard deviation (SD) of two independent grow up experiments. Wild-type specific activity is 66.91 ± 0.70. The *cnxC232* ^{ts} specific activity is 56.90 ± 2.06. The *cnxC465* ^{ts} specific activity is 59.69 ± 3.67.

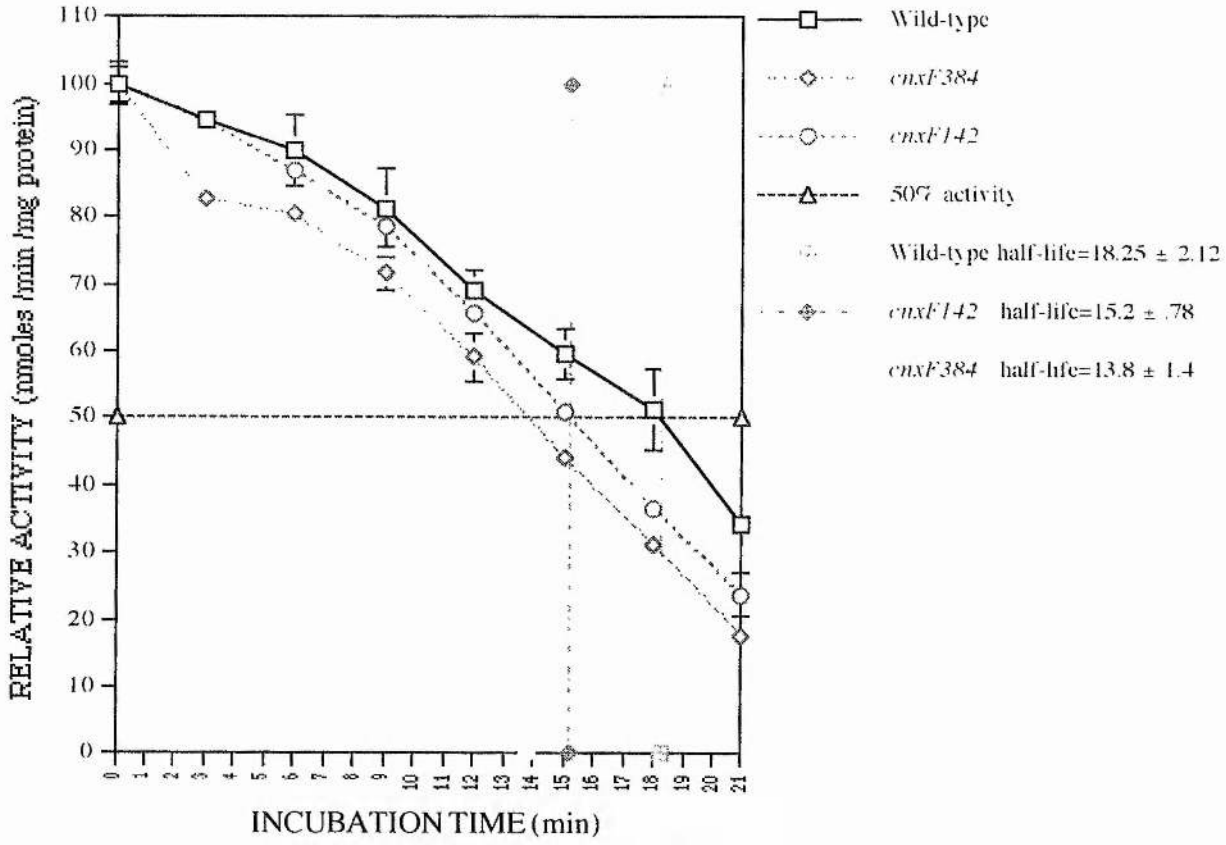


Figure 6.5. Relative Activities (specific activities normalised to the maximum specific activity at time =0) And Half-Lives At 35°C Of Nitrate Reductase Enzyme In The Wild-Type And Two *cnxF* Temperature-Sensitive Mutant (*cnxF142* ^{ts} and *cnxF384* ^{ts}) The wild-type and the mutants were grown for 21 h at 250 rpm, at 25°C in glucose minimal medium (with supplements), at pH6.5 with sodium nitrate (10 mM). Cell-free extracts were assayed at 25°C for 20 min. Soluble protein in extracts was determined using crystalline bovine serum albumin as standard (Bio-Rad protein standard). Enzyme activities are given as nanomoles of NADPH oxidised per min per milligram of protein. Values are mean ± standard deviation (SD) of two independent grow up experiments. Wild-type specific activity is 69.64 ± 3.17. The *cnxC232* ^{ts} specific activity is 58.79 ± 2.24. The *cnxC465* ^{ts} specific activity is 53.13 ± 2.62.

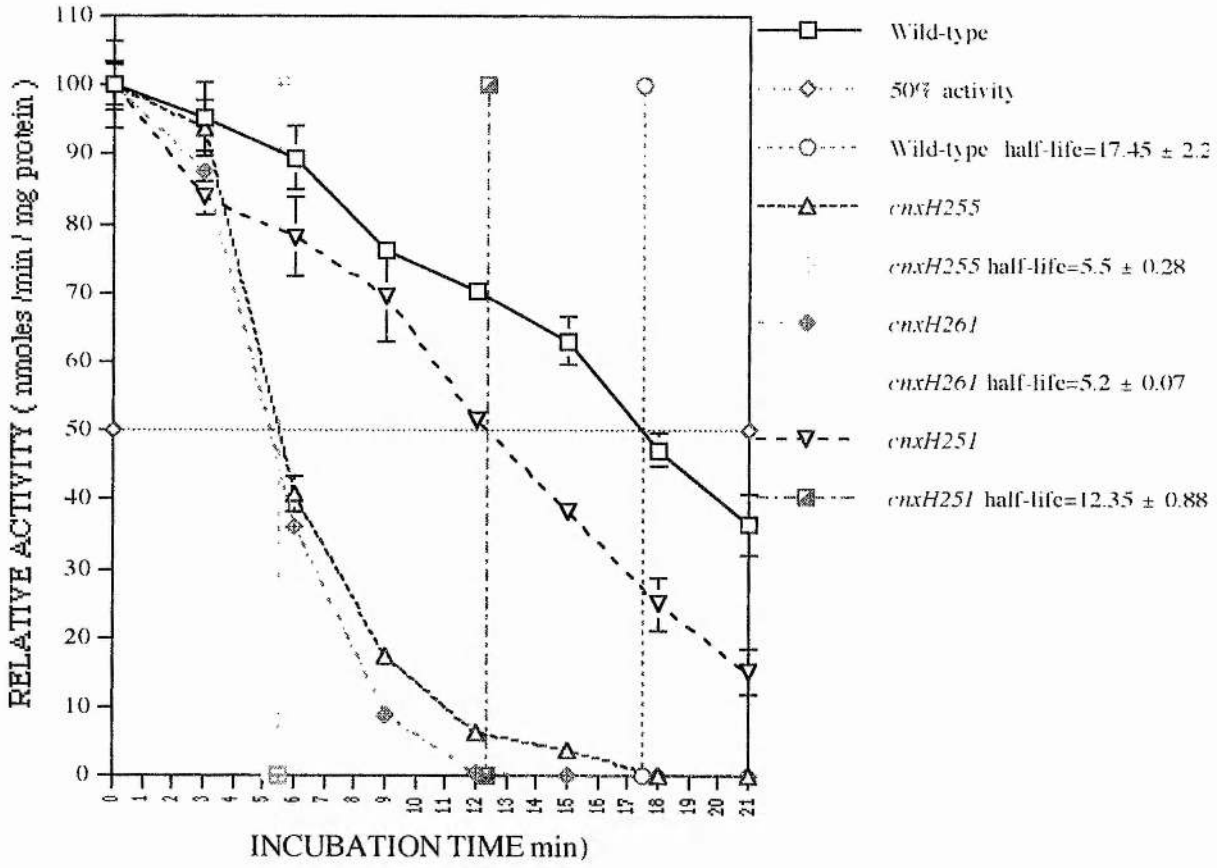


Figure 6.6. Relative Activities (specific activities normalised to the maximum specific activity at time =0) And Half-Lives At 35°C Of Nitrate Reductase Enzyme In The Wild-Type And Three *cnxH* Temperature-Sensitive Mutant (*cnxH251*^{ts}, *cnxH255*^{ts} and *cnxH261* ^{ts}) The wild-type and the mutants were grown for 21 h at 250 rpm, at 25°C in glucose minimal medium (with supplements), at pH6.5 with sodium nitrate (10 mM). Cell-free extracts were assayed at 25°C for 20 min. Soluble protein in extracts was determined using crystalline bovine serum albumin as standard (Bio-Rad protein standard). Enzyme activities are given as nanomoles of NADPH oxidised per min per milligram of protein. Values are mean ± standard deviation (SD) of two independent grow up experiments. Wild-type specific activity is 61.95 ± 6.30. The *cnxH251* ^{ts} specific activity is 53.09 ± 2.99. The *cnxH255* ^{ts} specific activity is 28.24 ± 3.50. The *cnxH261*^{ts} specific activity is 33.82 ± 1.02.

6.6 Molecular Analysis Of The *cnxH* Gene.

6.6.1 An Attempt To Isolate The *cnxH* By Chromosome Walking.

Attempts to isolate the *cnxH* gene was carried out by chromosome walking. In this regard the *meaB* gene maps 18 map units from *cnxH.* A cosmid containing *meaB* given to us by Dr. M. Caddick (University of Liverpool) was transformed into a *cnxH* strain. Unfortunately, no phenotypic complementation (i.e. growth on nitrate) occurred so the cosmid could not be carrying the *cnxH* gene. The *meaB* cosmid was used to isolate neighbouring cosmid clones from a chromosome III specific library. Twelve cosmids in the library hybridised to the original probe but surprisingly the probe did not hybridise to the cosmid carrying the same number given by Dr. Caddick i.e. W02E01 cosmid in the chromosome library. In addition *Hind* III digestion of both cosmids (i.e. W02E01 from Caddick and W02E01 from chromosome library) indicate that the two cosmids are totally different and unrelated to each other. It was decided that it is a matter of mistake in the cosmid number carrying the *meaB* gene and *cnxH* cloning procedures after this proceeded depending on this decision. The twelve cosmids that hybridised to *meaB* cosmid are as follows, W29A12, W19C12, W4B3, W4E6, L31C5, L6H3, L30G5, L19G1, L29E1, L29C8, L31F10 and L31F3.

DNA was prepared from each of these twelve cosmids and transformed twice into *cnxH4*. Only two cosmids appeared to complement the mutant (i.e. L31F10 and L31F3). Only three transformants were obtained with cosmid L31F10 in the first transformation experiment, whilst, cosmid L31F3 was generating one transformant in the first experiment, and two in the second. However, the physical (i.e. contiguous) map of chromosome three indicate that only two (W29A12, W19C12) of the twelve probed cosmids are in the map and these two are in an overlapping pattern. Furthermore, the stability of the generated transformants was tested by subculturing on selective and non-selective media and such transformants appear to be unstable i.e. did not grow on selective medium (i.e. nitrate) after growth on the non-selective due to loss of complementing DNA. This would suggest that these colonies are confirmed *Aspergillus* transformants.

Fungal DNA was prepared from the transformants and wild-type strain (see materials and methods section 2.3.1.5). The presence of bacterial sequences in putative fungal transformants was confirmed by the ability to amplify such sequences using the PCR method (see materials and methods section 2.4.2). The choice of primers needed was dependent on the clone used. In these cosmid transformants fungal DNA was amplified using λ1 and λ2 primers (see materials and methods section 2.4.2.3). In these cases, no PCR products were generated from the transformants DNA sequences, however both positive (wild-type) and negative (SDW) were

included in the PCR reactions. The negative result obtained by the PCR indicate that these were not real transformants and they might be either revertants or contaminants.

Furthermore, according to the position of the two overlapping cosmids (i.e. W29A12 and W19C12) on the physical map, six cosmids from each direction were selected as a first walk along chromosome three. The list of these cosmids as follows, W8E8, W8C4, L23D11, W9A8, L4E2, W18E4, W4E6, L8B8, W23D12, W23D3, L24F12 and W6A10. The DNA was prepared from these cosmids and transformed into the *Aspergillus cnxH4*. Four different experiments were performed and in each experiment six cosmids were transformed in. In all transformation experiments, only one cosmid, W8E8, appeared to complement the mutant. A fungal DNA mini preparation of the putative transformant was carried out and a PCR reaction was performed using ampicillin primers (see materials and methods section 2.4.2.4), in which no PCR product was obtained suggesting that the isolate was a revertant rather than a true transformant.

The *meaB* cosmid was tested and transformed with the a *meaB cnxH* double mutant the obtained results indicated that the cosmid is carrying *meaB* gene but not *cnxH* gene. In addition the cosmid W02E01 from chromosome III library was also tested but it did not complement any of the mutations which would indicate that the provided cosmid is the right one but with a wrong number.

Such confusing results lead to the suggestion that all predicted fungal transformants were not confirmed ones. Instead these could be either contaminants or revertants. However, the explanation for such confusion could be related to different reasons. First, chromosome three cosmid library might be made from at *cnxH* mutant strain originally. Second, the number of clones in the bank do not cover the whole chromosome. Third, *cnxH* transformation might cause an increase in the copy number of other unrelated gene(s). The product(s) of such gene or the product of the activity of the gene could inhibit transformant cells thus successful transformation would not eventuate.

6.6.2 A Second Attempt For The Isolation Of The *cnxH* Gene Using Genomic DNA Or argB Based Genomic Bank Along With Replicating Plasmids.

During the confusion of chromosome walking it was decided to carry out gene cloning by using autonomously replicating plasmids (described in the introduction see Figure 1.5). Several fungal self-cloning transformation experiments were carried out using as the host either the single (i.e. *cnxH4*) or double mutants (i.e. *argB, cnxH4*). In these experiments either the argB genomic bank or wild-type genomic DNA (partially digested with the restriction endonuclease *Sau* 3A) were used along with a helper plasmid to transform *Aspergillus* mutant strains. Table 6.5 shows the transformation frequencies obtained with these strains. The stability of the generated transformants was tested by subculturing on

selective and non-selective media and such transformants appear to be stable i.e. did not grow on selective medium (i.e. nitrate) after growth on the non-selective due to the loss of complementing DNA. Subculturing of transformants from the transformation plate imediately to another nitrate plate showed unstable growth.

Fungal DNA was prepared from putative *A. nidulans* transformants and rescued in *E.coli.* Plasmid pools were made from all *E. coli* transformants. Such pools of DNA were numbered as indicated in Table 6.5. Furthermore, these pools were re-transformed into strain *cnxH4* and Table 6.6a shows the retransformation frequencies obtained with these pools. In addition, pools of wild-type DNA that have been transformed with the markerless plasmid pHELP were divided into subpools according to the original *Aspergillus* transformant (i.e. each group of *E. coli* transformants obtained from a single *Aspergillus* transformant was considered as subpool). Prepared DNAs from subpools were transformed with the strain *cnxH4* and Table 6.6b shows the retransformation frequencies obtained. However, before proceeding to further create pool subdivisions, it was decided to use pHELP as a hybridisation probe in Southern blots with these positive subpools in order to determine if there is any 'extra' *Aspergillus* DNA present on these plasmids. DNA hybridisation of these subpools with pHELP as the probe indicate the presence of extra bands (extra' with regard to pHELP) of DNA. However such 'extra' DNA fragments

appeared too small to contain a gene. Therefore, it was decided to use only the pHELP system and dealing with *E. coli* rescued transformants as single preparations rather than pools.

6.6.3 The Isolation Of The *cnxH* Gene Using The pHELP System.

Either wild-type DNA (partially digested with *Sau* 3A) or the argB genomic bank with pHELP (cleaved or uncleaved with *Bam*HI) were used for *cnxH* gene cloning experiments. The cloning strategy mentioned in Figure 2.1 summarises the gene isolation steps. Another *cnxH* strain was used in this attempt (*cnxH3*). Transformation frequencies obtained with the strain are shown in Table 6.7. In addition, the results in Table 6.7 show the total number of *Aspergillus* transformants that have been subcultured into nitrate media from the original transformation plates. The data in this Table also shows the total number of confirmed transformants which were subcultured from nitrate to a further nitrate plate. The subculturing of transformants showed that such transformants were growing with different rates and looked morphologically irregular (i.e. unstable growth). However, the stability of transformants was tested by subculturing on complete (i.e. non-selective media). After growth it was transferred to nitrate media (i.e. selective media again). All transformants selected with the pHELP plasmid were unstable i.e. did not grow on selective media after growth on non-selective due to loss of complementing plasmid on non-selective media.

Isolation Of DNA From *A. nidulans* Complementing *cnxH3* Transformants And Rescuing Of Complementing plasmids By Transformation Of *E. coli.*

Genomic DNA was isolated from three likely *Aspergillus* transformants (indicated in Table 6.7 using the method described in section 2.3.1.5) of these 10 μl DNA was used to transform *E. coli* competent cell strain DH5$\propto$. Only one Aspergillus transformant DNA preparation yielded E. coli transformants. Six *E.coli* transformants, from a total of 14 obtained, were cultured on fresh Luria plates with ampicillin (for selection). Such *E.coli* colonies, on the purification plates, were variable in size. Two colonies in each size range were chosen and each colony was subcultured onto fresh Luria containing ampicillin. Cracking the cells itself (quick plasmid preparation, see materials and methods) indicated that certain colonies were not containing any plasmid. Four plasmids rescued designated p5.2, p5.4, p5.5 and p5.6 had fragments (i.e. *Ecor* I digestion) larger than pHELP).

Retransformation Of *cnxH3* With Rescued Plasmids In *E. coli.*

Plasmid DNA was prepared from plasmid clones p5.2, p5.4, p5.5 and p5.6. These plasmids were retransformed into *cnxH3* and Table 6.8 shows the frequencies obtained in these *Aspergillus* re-transformation experiments. High frequency transformation was observed with the designated plasmids p5.6 , p5.5, or p5.4 . Plasmid DNA preparation from p5.6 or p5.5 was carried out and both

plasmids were checked again by retransformation into *Aspergillus pantoC3 Sc12 cnxH3* strain (Table 6.8). The two plasmids were digested with the endonuclease *EcoR* I, in which the digestion indicate the clear presence of extra DNA bands (i.e. other than pHELP bands) (Figure 6.7).

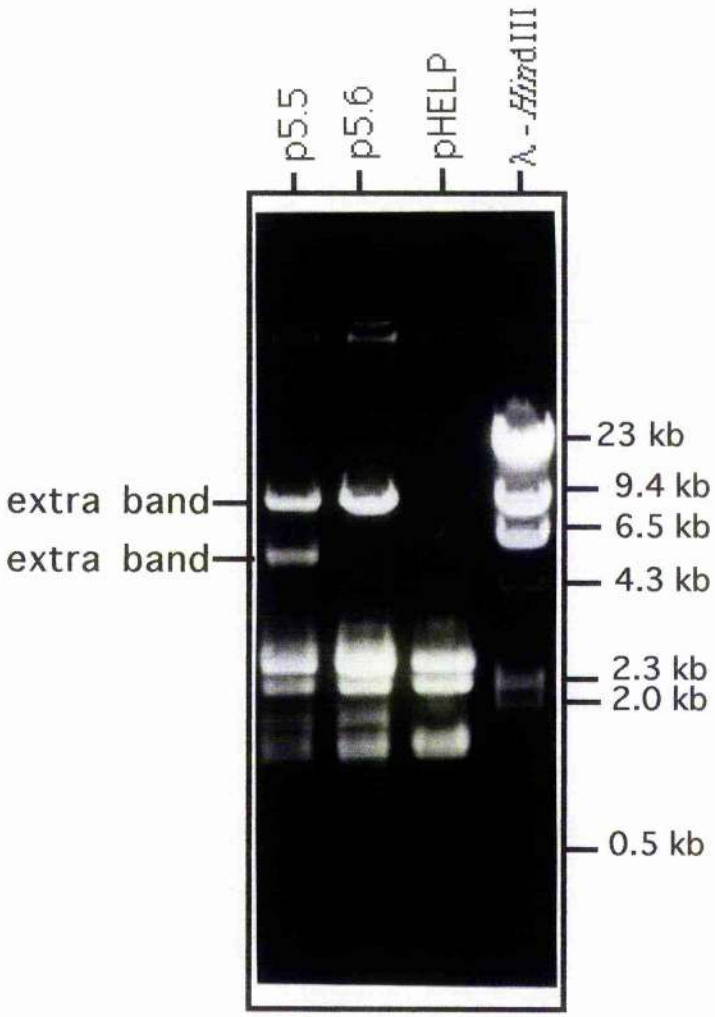
In order to determine more definitely if there are *Aspergillus* 'extra' DNA bands in these plasmids, such plasmids were digested with further enzymes i.e. *Hin* dIII, *Bam* HI, *Eco* RI and Southern blotted using vector pHELP, digested with *Eco* RI as the probe. Southern blot results indicate that *Bam* HI digestion of recombinant plasmid p5.6 possesses 'extra' (0.75 kb and 3.5 kb) bands that did not hybridise to pHELP sequences (Figure 6.8).

Using the gene clean method (see materials and methods) both 0.75 kb and 3.5 kb *Bam* HI fragments were isolated from a 2% agarose gel (Figure 6.9) and were used (20 ng/μl) individually to probe cosmids from the chromosome three specific gene library.

6.6.4 Southern Blot Hybridisation Against Chromosome III Cosmid Bank.

Both *Bam* HI fragments of plasmid p5.6 were labelled with 30 μCi of $\propto$ -32 p dCTP and hybridised to chromosome III cosmid bank library. Both probes hybridised to seven cosmids and the 3.5 kb probe to one other cosmid (Figure 6.10), suggesting that the fragments used as probes are contiguous. Digests of the cosmid DNAs clearly show common fragment sizes (Figure 6.11).

Figure 6.7. *Eco*RI digestion of two rescued plasmids in *E. coli* transformation using fungal isolated DNA. Both rescued *E. coli* plasmids possessed 'extra' DNA fragments.



Eco R I digestion

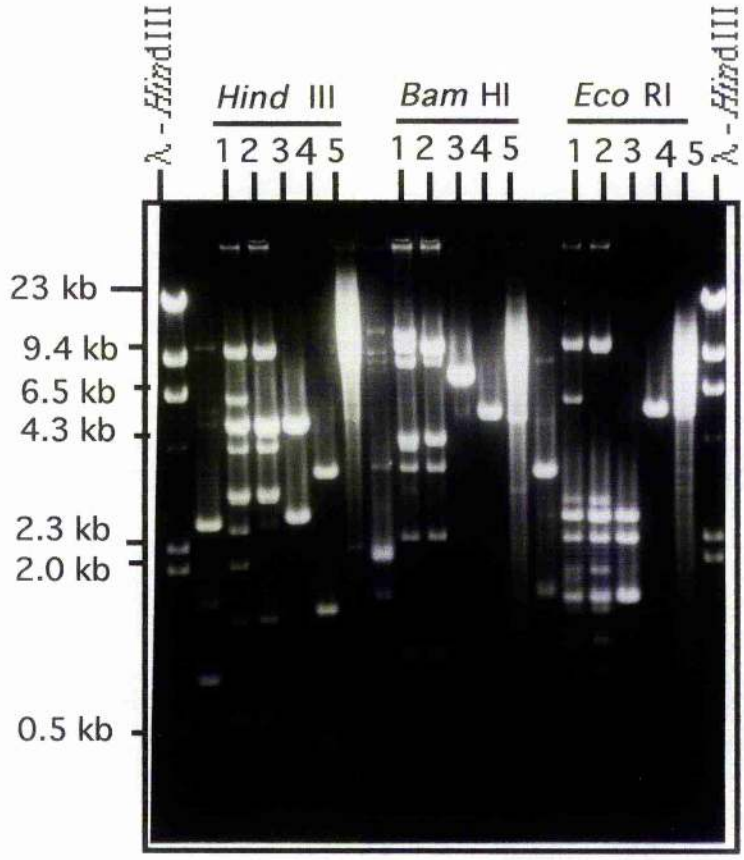
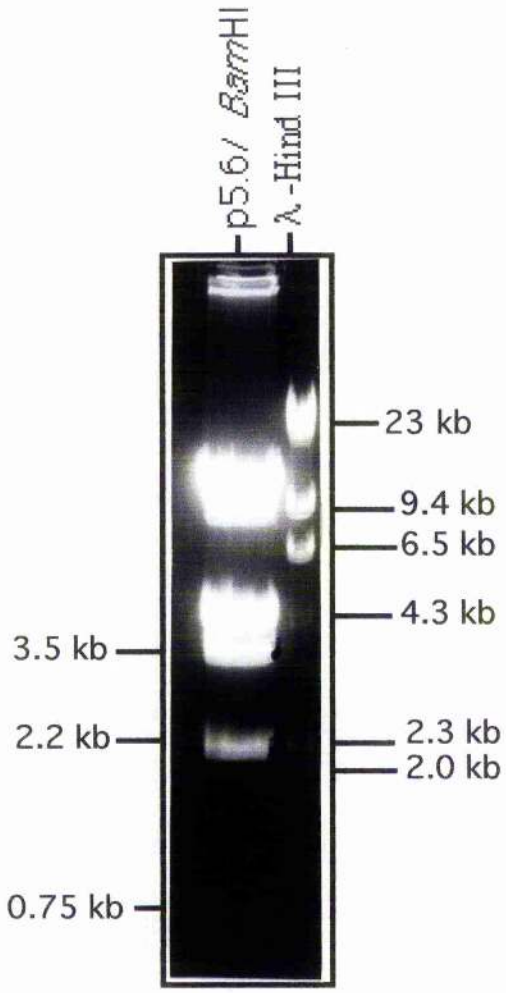


Figure 6.8. *Bam* HI, *Hin* dIII and *Eco* RI digestion of the *E. coli* rescued plasmids p5.5 and p5.6, along with vectors pHELP, pILJ16 and *A. nidulans* argB bank. This was Southern blotted using *Eco* RI digested pHELP as the probe. The Southern blot indicated that *Bam* HI digestion of the plasmid p5.6 revealed 'extra' bands, that did not hybridise at all to pHELP sequences. The size of these bands is 0.75 kb and 3.5 kb. Number 1 denotes recombinant plasmid p5.5, 2: denotes p5.6, 3: denotes vector pHELP. 4: denotes pILJ16, 5: denotes the argB bank.

Figure 6.9. *Bam* HI digestion of plasmid p5.6 showing 'extra' bands which did not hybridise to vector pHELP sequences. The molecular weights of these bands are 0.75 kb and 3.5 kb.


p5.6/ BamHI
λ-Hind III
23 kb
9.4 kb
6.5 kb
4.3 kb
3.5 kb
2.2 kb
2.3 kb
2.0 kb
0.75 kb

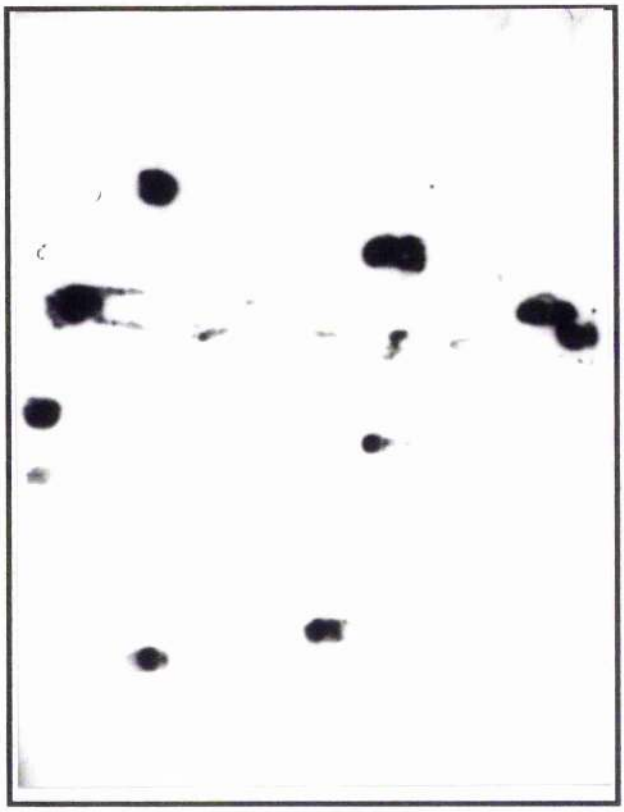


Figure 6.10. Southern blot hybridisation against chromosome 3 specific library. Eight cosmid clones hybridised to both *Bam* HI fragments obtained from recombinant plasmid p5.6.

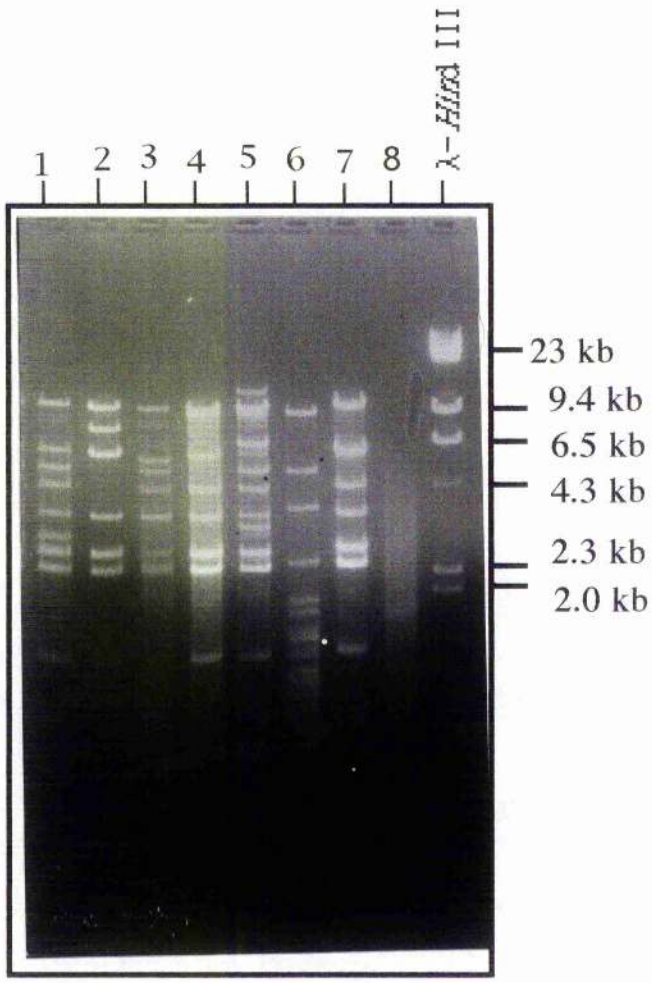


Figure 6.11 Agarose gel electrophoresis of Eco RI digests for 7 cosmid clones in chromosome 3 specific library that hybridised to both Bam HI fragments (i.e. 0.75 kb and 3.5 kb) of plasmid p5.6. Lane 1 cosmid L19G10. Lane 2 cosmid L25O3. Lane 3 cosmid L30C1. Lane 4 cosmid W1E8. Lane 5 cosmid W5B8. Lane 6 cosmid W6O5. Lane 7 cosmid W22B10. Lane 8 cosmid W23E4 (cosmid in lane 8 was prepared again).

Fungal Transformation With Cosmid Clones.

All eight positive clones were transformed into the *cnx* strains in two separate experiments each with four clones in addition to the original complementing plasmid 5.6. In each experiment the plasmid was transforming strains *cnxH3* or *cnxH4* at high frequency ranging between 2000 to 5000 transformants, whilst none of the eight cosmids has generated a single transformant in any of the experiments. In all cases protoplasts viability was more than 1 x 10^7 /ml. The eight cosmid clones used in the transformation are listed below, L19G10, L25O3, L30L11, W1E8, W5B8, W6O5, W22B10 and W23E4. Since none of the cosmid clones has complemented *cnxH* this could be related to the same reasons discussed before in section 6.6.The plasmid p5.6 digested with the restriction endonucleases *Hind* III, *Bam* HI, or *EcoR* I was transformed into strain *cnxH4* the retransformation frequencies are shown in Table 6.9. These results indicate that both *Hind* III and *EcoR* I fragments were transforming *cnxH* 4 mutant at high frequency as compared to *Bam* HI digests.

The Isolation Of *Eco* RI And *Hin* dIII DNA Fragments From Recombinant Plasmid p5.6.

Ten bands were visible in *Eco* RI digests, whilst seven bands were cut with *Hin* dIII (Figure not shown). However, not all bands are just one fragment. In addition faint bands between *Eco* RI 4.4 kb and 6.6 kb and *Hin* dIII 9.5 kb and 23 kb were not isolated. DNA isolated from five different *Eco* RI digest bands along with

the original p5.6 vector as well as pHELP were transformed into *cnxH4* strain. Transformation frequencies obtained indicate that only band 9 (approxi 3.5 kb) complemented this mutation (i.e. greater than 1000 nitrate utilising transformants). The original plasmid (p5.6) was used as a positive control and over 5000 nitrate utilising transformants were obtained (Figure 6.12). The complementing *Eco* RI band (i.e. band 9) was ligated to the common vector pUC18 (100 ng/μl) and recovered by *E. coli* transformation. The isolated *Eco* RI band 9 of plasmid p5.6 but not the pUC18 clone was used as probe for hybridisation with the argB genomic bank. DNA was prepared from the positive hybridising argB clones (namely arg3/3, arg4/2, arg5/1, and arg5/2) and transformed into *cnxH4* strain. Table 6.10 shows the transformation frequencies obtained with these clones together with the control.

Fragment Isolation.

The results presented in Table 6.10 indicate that two argB recombinant clones (i.e. argB3/3 and argB5/1) showed high transformation frequencies. Both clones were digested with *Eco* RI and Figure 6.13 shows the sizes of the cleaved fragments for both clones. Such fragments (from both clones) were isolated and re-transformed with the *Aspergillus* strain *cnxH4*. Table 6.11 shows the retransformation frequencies obtained with these subclones and indicate that the *Eco* RI fragment 1.9 kb of the argB5.1 clone gave the highest transformation frequency.

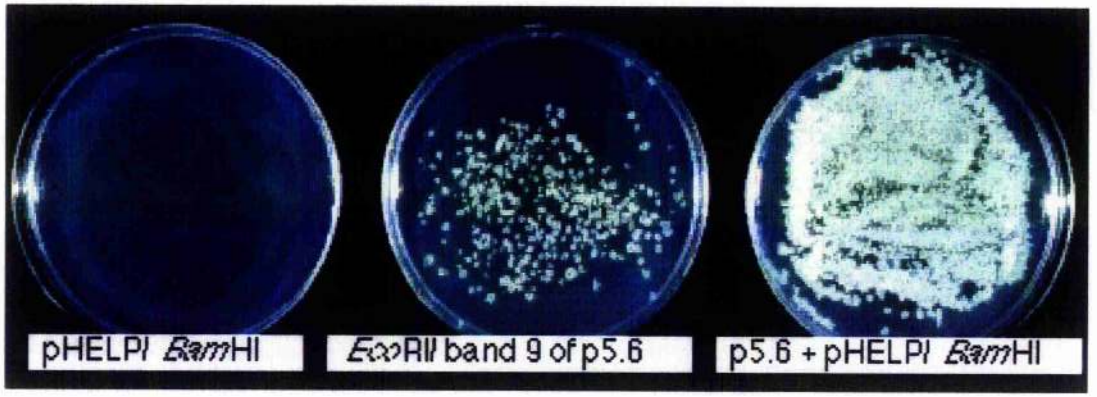
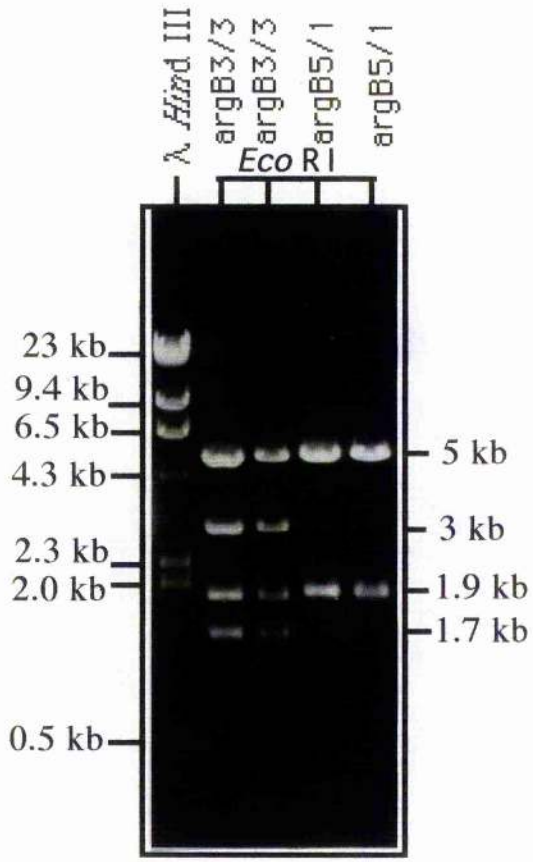


Figure 6.12 Complementation of *cnxH* mutation by an *Eco* RI fragment of the rescued plasmid p5.6. DNA isolated from an *Eco* RI fragment (i.e. band 9) of the rescued plasmid p5.6 along with the plasmid as well as pHELP were transformed into *cnxH4* strain. pHELP cleaved with *Bam* HI was used as a negative control whilst, the plasmid p5.6 as a positive control. Transformation results indicated that *Eco* RI band 9 of p5.6 plasmid had complemented *cnxH* mutation at high frequency.

Figure 6.13. *Eco*RI digests of two argB clones (i.e. argB 3/3 and argB 5/1). Retransformation results with each fragment from both clones indicate that the 1.9 kb fragment of clone argB 5/1 was giving the highest transformation frequency.


λ Hind III
argB3/3
argB3/3
argB5/1
argB5/1
Eco RI
23 kb
9.4 kb
6.5 kb
4.3 kb
2.3 kb
2.0 kb
0.5 kb
5 kb
3 kb
1.9 kb
1.7 kb

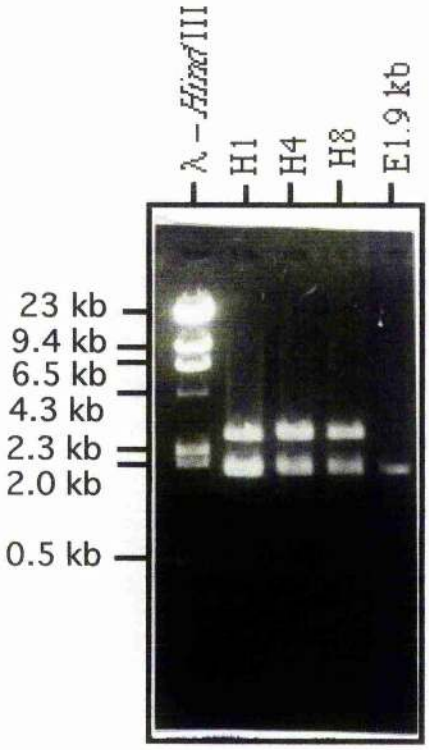
The Isolation Of DNA Subclones..

The 1.9 kb *EcoR* I fragment was ligated to the vector plasmid pUC18 digested with *Eco* RI (approx 100 ng/μl). Quick DNA (see materials and methods) preparation of ligated DNA indicated the presence of three subclones designated H1, H4 and H8. Subclone H1 showed a more heavily stained band which indicates the presence of two copies of the 1.9 kb fragment. DNA prepared from subclones H1, H4 and H8 was digested with *Eco* RI (Figure 6.14). The three subclones along with the argB5/1 clone were transformed with *cnxH4* strain. The transformation frequency of these subclones is presented in Table 6.12. Subclone four which transforms *Aspergillus* at high frequency was ligated with the vector pUC18 (i.e 1.9 kb *Eco* RI fragment in pUC18). The entire wild-type 1.9 kb fragment that was likely to the *cnxH* gene has been sequenced along with non-conditional *cnxH* mutants. Assume this is pSTA509.

6.6.5 DNA Sequence Analysis.

Sequence data and genetic alignments were stored and manipulated on Apple Macintosh computer *via* the gcg programme (Daresburge, U.K). sequence analysis were carried out using BLAST programme (National Centre for Biotechnology Introduction) available on Internet (NetScape Navigator 2.0).

Figure 6.14. The three subclones designated H1, H4 and H8 represent the 1.9 kb *Eco*RI fragment of plasmid argB 5/1 ligated to pUC18 digested with *Eco*RI. In each subclone the upper band is the pUC18 vector and the lower band is the 1.9 kb *Eco*RI fragment. The band in subclone H1 is ~~larger~~ more heavily fluorescent than the other two subclones indicating the presence of two copies of the 1.9 fragment.


λ – HindIII
H1
H4
H8
E1.9 kb
23 kb
9.4 kb
6.5 kb
4.3 kb
2.3 kb
2.0 kb
0.5 kb

Sequence Determination Of The *cnxH* Gene.

Both strands of the subclone PSTA509 were sequenced by the di-deoxy chain termination method as described in materials and methods. The DNA sequencing strategy used is shown in Figure 6.15. The entire sequence of the 2 kb *cnxH* subclone as well as the deduced amino acid sequence are presented in Figure 6.16. The open reading frame has a length of 585 bp encoding a protein of 195 amino acid. A 1033 bp leader sequence of the 5' region, upstream of the start codon, and the 153 bp long, 3' untranslated trailer region (down stream) was sequenced.

6.6.6 DNA Sequence Analysis Of Temperature Conditional And Non-Conditional *cnxH* Mutants.

Certain primers used in the sequencing of the wild-type *cnxH* gene were used in PCR reactions for amplifying DNA fragments from different mutant alleles of the *cnxH*. These PCR products were sequenced to positive identity confirm the genomic sequence of the gene. In each PCR reaction either wild-type DNA (as a positive control) or no DNA (i.e. double autoclaved sterile water as a negative control) were included.

Sequence Changes In The *cnxH261* ^{ts} Mutant.

Complete sequence analysis of the coding region of mutant PCR DNA indicates that the *cnxH261* ^{ts} mutation is due to a 3 base pair insertion (i.e. a glycine codon) at position + 443 bp (Figure 6.16) from the ATG start codon. This mutation lead to the

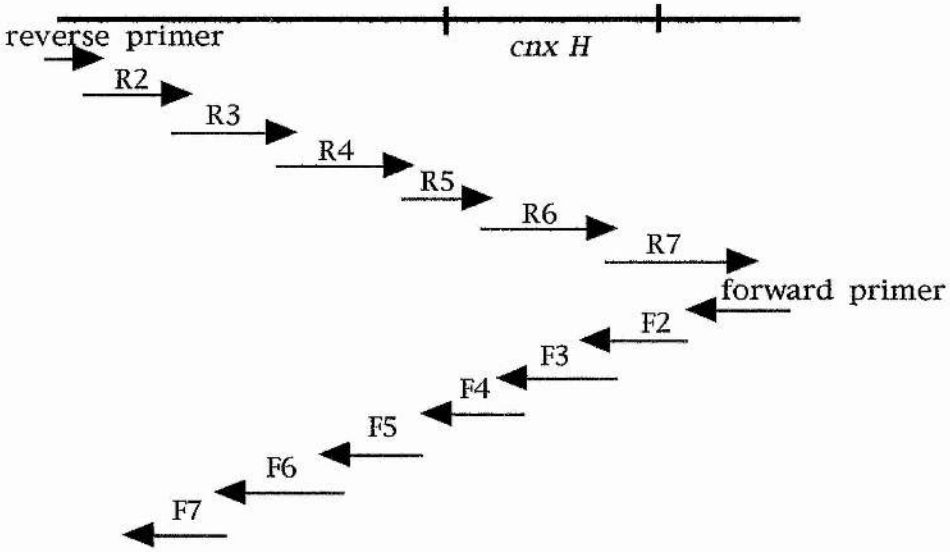


Figure 6.15. The DNA sequencing strategy adapted for nucleotide characterisation of the region containing *Aspergillus nidulans cnxH* gene within the Psta509 subclone. Sequencing was carried out on both nucleotide strands.

Figure 6.16 DNA sequence of the *Aspergillus nidulans* *cnxH* gene. Nucleotide and amino acid sequences of the region of *A. nidulans* genomic DNA that transforms *cnxH* to wild-type. The highlighted regions showed The *Hind* III-*Xba* I fragment of the coding region. No homologies with other genes were found within the upstream region.

cnxH **wild-type**

```
-1033   GGGAGCCCACTCTGGTCGGCGGAGTGCCAGCGTGGGTTCCGTATGATCGCAGGACTCCCT

 -973   CGCCGGGATCCCGCTCGGCCTCCAACTAGAGACGGCCCTTACAGCGGACGGTAAAGGAAG

 -913   CCAAGGGAGGAAGATGGTATGGTGATATTCCGATTTCGCCTGTCGGAACCGATCCTTTCA

 -853   TTGAAGGGCAGTCTGGAGTTCAAGAGGCGACTCCTGTCACTGGCTCCCAGAGCCAAGGAC

 -793   ATTACGCCAGCTATTATGAGCCAGAGAGCACCTACCCTCCACGCACCACTGCCGGACATG

 -733   AAGCCAGCATGGGCAGCAGCGAGCCTGGCGATGACTATGGTTACCAGCAAACCTCGCAAA

 -673   GCCGCCAGATGGCCTACCCCCTGTCAGAAGAGAGCGACGAGTATGAGCAAGCGTCGCTCG

 -613   AGGATAGTGAAGGCGGCTATGGACAAGTACATGCCTCCGACGTCTGGGCCAATGTACGCT

 -553   GGTTACGACCAGTCAGTCGACTACAGCGGACGCAGCTGGGGCGTCGGGACCGGCTGGGAA

 -493   TCTGTGACTCCCCGCCATCGCCATCCGACGCGCCTTAGGCATGTCCTGGAAGAGGAGGAG

 -433   CCCCGGTCGACCCCCAGCCGAGCTAGCCGTGCCAGCCAGGCGAGCAGGAGTGTTCAATGA

 -373   ACACCGGTATATGACTTCTTCATTTTTGGATTTCTATTTGATATGCCATTGATCATATGC

 -313   GGGTTTTATATTTATGTTCTCCCAGTTCTTTTCAGTTTCTCGCCCGCGATACTCCAACCG

 -253   CTTTCTTCTCAGTTCAGTCAGTTATTGTCATTGTTTCAGCATATAGACTCGGAGCAGGAA

 -193   GCGGGAGTAATCTGTAATTCTAGTGATGATACGACCGTGGATATACGCCTCAATGAAAGT
                                                          Hind III
 -133   CTTTTGCAGATACAGAAACCAAATTTCGTTATCGGTCGCAGCCTGAGGCAGTGCAGTTCA|

  -73   AGCTTATCAGTTCCCCTCAACAGATCGGTCTCTCCACCAGCATCTCATACACAAACAACC

  -13   ACACCCACATACAATGTCAGCCAGACCCGAACCGCAACCGGGATCCGAACGCAACGCAAC
                      M  S  A  R  P  E  P  Q  P  G  S  E  R  N  A  T

   47   AGAACCCCTCCCTTCACACCTTGACCCAACAACCTATCCGCGAACCCTCACAACAACTCA
         E  P  L  P  S  H  L  D  P  T  T  Y  P  R  T  L  T  T  T  H

  107   CGGGCCCACCAGCATTCCCCTCCACCTCGAGCTAACATACCACACGCTCTCCCCCACCAC
         G  P  T  S  I  P  L  H  L  E  L  T  Y  H  T  L  S  P  T  T

  167   CGCCCTGCAACACGTTTCCTCCCCTAGTAGCGGTGCAAACATTCTCTTCCTCGGCACAAC
         A  L  Q  H  V  S  S  P  S  S  G  A  N  I  L  F  L  G  T  T

  227   ACGCGACACGTTTGATGACCGTCCCGTCGCCCGCCTCTCATATACATCATACCCAGCCCT
         R  D  T  F  D  D  R  P  V  A  R  L  S  Y  T  S  Y  P  A  L

  287   CGCGCTGAAATCATTGCACAAAATCTCCTCTGAAGCGGTTGAGAAGTTCGGGCTGAATGG
         A  L  K  S  L  H  K  I  S  S  E  A  V  E  K  F  G  L  N  G

  347   TGTCTATATCGCGCATCGGCTTGGTGAAGTTCCCGTTGGGGAGGCGAGTATTGTTGTTGC
         V  Y  I  A  H  R  L  G  E  V  P  V  G  E  A  S  I  V  V  A

  407   GGTGGGGGCGGGGCATAGAGGGGAGGCCTGGAGGGGCGCGGAGTGGGTGCTTGAGGTTGT
         V  G  A  G  H  R  G  E  A  W  R  G  A  E  W  V  L  E  V  V

  467   GAAGGAGCGGGTTGAGGTGTGGAAGAGGGAGGAGTTTGTGGATGGAGGGATGGAGTGGAG
         K  E  R  V  E  V  W  K  R  E  E  F  V  D  G  G  M  E  W  R
```

Xba I

527 GGAGAACAGGGAGAGGGATGGGTTTGGAAAATTGAAGACCAAAAAGGAGGATT|**CTAG**ATG

E N R E R D G F G K L K T K K E D S R

587 ATTGGAGAGCTATGGTACAAGACTTGACAGGATGGATATTATAAATACACGAATGGTTGC

647 TTGGACGAGGAAAGGCAACATTCTAATCCCTCTTTAAGGGGCTTTCCCTCTCTCTTCCAG

707 TCATTGATATGCTCCATGATCGTCTTCGTCGCGG

temperature sensitivity of the NR enzyme i.e. approximately 40% reduction in the half-life of the mutant enzyme as compared to the wild-type.

Sequence Changes In The *cnxH427* Mutant.

Complete sequence analysis of the coding region of mutant PCR DNA indicates that the *cnxH427* mutation is due to a single bp substitution (i.e. G changed to A) at position + 453 bp (Figure 6.16) relatively to the ATG start codon. This mutation lead to premature termination of the protein and the resulting small translated protein is non-functional in such strain. This premature protein lead to inability of the mutant to utilise either nitrate or hypoxanthine (the substrates for the three molybdoenzymes NR, PH I and PH II) as the sole nitrogen source. Loss of activity of these enzymes suggested to be due to the lack of Mo-Co biosynthesis, where the suggested role of the *cnxH* gene in this biosynthetic pathway is specifying a protein or polypeptide structurally involved in the molybdoenzymes.

Sequence Changes In The *cnxH911* Mutant.

The mutation in strain *cnxH911* was found to be due to a single bp insertion (namely T) at nucleotide position + 245 bp (Figure 6.16). This mutation lead to a shift in the reading frame to another frame and premature termination of the protein at nucleotide position + 318 bp.

6.6.7 Data Base Searches.

The nucleotide sequence of the gene was analysed using the Macintosh computer. Using the BLASTX programme, peptide sequences which showed homology with the sequence in question were obtained.

cnxH Protein Homologues.

The *cnxH* protein sequence showed similarity with *moaE* gene products of *E.coli* which is involved in the regulation (i.e. converting the large subunit into active MPT) of the converting factor (MPT synthase) which converts the precursor into molybdopterin (MPT). and *moaE* gene products of *H. influenza*. The deduced *cnxH* amino acid sequence showed approximately 28% identity and 50% similarity to the corresponding region from *E. coli moaE* (Figure 6.17a). In addition, the deduced amino acid sequence of *cnxH* showed approximately 27% identity and 59% similarity to the corresponding region of the *moaE* gene product of *H. influenza* (Figure 6.17b). However, a comparison between the *E. coli* and *H. influenza* deduced amino acid sequence (Figure 6.17c) showed only 57% identity and 70% similarity. This amino acid comparison indicates that both bacterial sequences are not as similar as their identity to the *cnxH* product of *Aspergillus nidulans*. These observations would suggest that the *Aspergillus cnxH* gene probably has the same function as *moaE* gene products in bacteria.

Figure 6.17. Comparison Between The Deduced Amino Acid Sequences In The *Aspergillus nidulans cnxH* And The Bacterial *moaE* Genes Of *E.coli* And *H. influenza.*

In (a) protein homology in both bacterial genes showed approximately 57% identity and 70% similarity.

In (b) the protein homology in *cnxH* and the *moaE* gene of *E.coli* showed approximately 29% identity and 50% similarity.

In (c) the protein homology in *cnxH* and the *moaE* gene of *H. influenza* showed approximately 27% identity and 59% similarity.

Symbole (|) denotes identical amino acids.

symbol (:) denotes similar but not identical amino acids.

moaE of *E. coli* against *moaE* products of *H. influenza* .

```
  1 AETKIVVGPQPFSVGEEYPWLAERDEDGAVVTFTGKVRNHNLGDSVNALT  50 E.coli
    .:..|.|..|||. .. |.||.|... ||.|.|.|||| |||.|..|
  2 TDIQIAVQEQPFDQNAVYQWLSEQHSIGATVIFVGKVRDLNLGDEVSSLY  51H. influenza
 51 LEHYPGMTEKALAEIVDEARNRWPLGRVTVIHRIGELWPGDEIVFVGVTS 100 E.coli
    |||||:||||||.|||.:|: ||.:.||.||||:| | .|||||:|||.|
 52 LEHYPAMTEKALNEIVAQAKVRWDIQRVSVIHRVGLLQTGDEIVLVGVSS 101 H. influenza
101 AHRSSAFERGQFIMDYLKTRAPFWKREATPEGDRWVEARESDQQAAKRW 149 E.coli
    |||:.|:. .:||||:||.:|||||:|.| :|:||:||||||.:| .:|
102 AHRGDAYHANEFIMDFLKSKAPFWKKEQTNQGERWIEARESDKEALEKW 150 H. influenza
```

a

Aspergillus nidulans cnxH. against *moaE* products of *E.coli*

```
 33 TTTHGPTSIPLHLELTYHTLSPTTALQHVSSPSSGANILFLGTTRDTFDD 82 cnxH
    .|. .... |: :: .|. |.        .....|| : | |..|:   :
  2 ETKIVVGPQPFSVGEEYPWLA.........ERDEDGAVVTFTGKVRNHNLG 43 moaE
 83 RPVARLSYTSYPALALKSLHKISSEAVEKFGLNGVYIAHRLGEVPVGEAS 132 cnxH
     .|. |... ||::. |.| .| .|| :::.|. | : ||:||: .|:.
 44 DSVNALTLEHYPGMTEKALAEIVDEARNRWPLGRVTVIHRIGELWPGDEI 93 moaE
133 IVVAVGAGHRGEAWRGAEWVLEVVKERVEVWKREEFVDGGMEWRENRERD 182 cnxH
    :.|:|..:||:.|:  :::::: :|.|...|||. .:|:. | |.||.|
 94 VFVGVTSAHRSSAFERGQFIMDYLKTRAPFWKREATPEGDR.WVEARESD 142 moaE

183 GFGKLK 188 cnxH
    . :  :
143 QQAAKR 148 moae
```

b

Aspergillus cnxH against *moaE* products of *H. influenza* .

```
 41 IPLHLELTYHTLSPTTALQHVSSP.SSGANILFLGTTRDTFDDRPVARLS 89 cnxH
    ..::::.:  :.:.....| :|.. | ||.::|:|..||   : .|..|
  2 TDIQIAVQEQPFDQNAVYQWLSEQHSIGATVIFVGKVRDLNLGDEVSSLY 51 moaE
 90 YTSYPALALKSLHKISSEAVEKFGLNGVYIAHRLGEVPVGEASIVVAVGA 139 cnxH
    .. |||:. |.|:.| .:|  ::::. | : ||:| :..|:. ::|:|:.
 52 LEHYPAMTEKALNEIVAQAKVRWDIQRVSVIHRVGLLQTGDEIVLVGVSS 101 moaE
140 GHRGEAWRGAEWVLEVVKERVEVWKREEFVDGGMEWRENRE..RDGFGKL 187 cnxH
    :|||:|:::.|:::::.:|.:...||:|: .:.|  | |.||  :::::|:
102 AHRGDAYHANEFIMDFLKSKAPFWKKEQ.TNQGERWIEARESDKEALEKW 150 moaE
```

c

6.6.8 Northern Blot And The Regulation Of Expression.

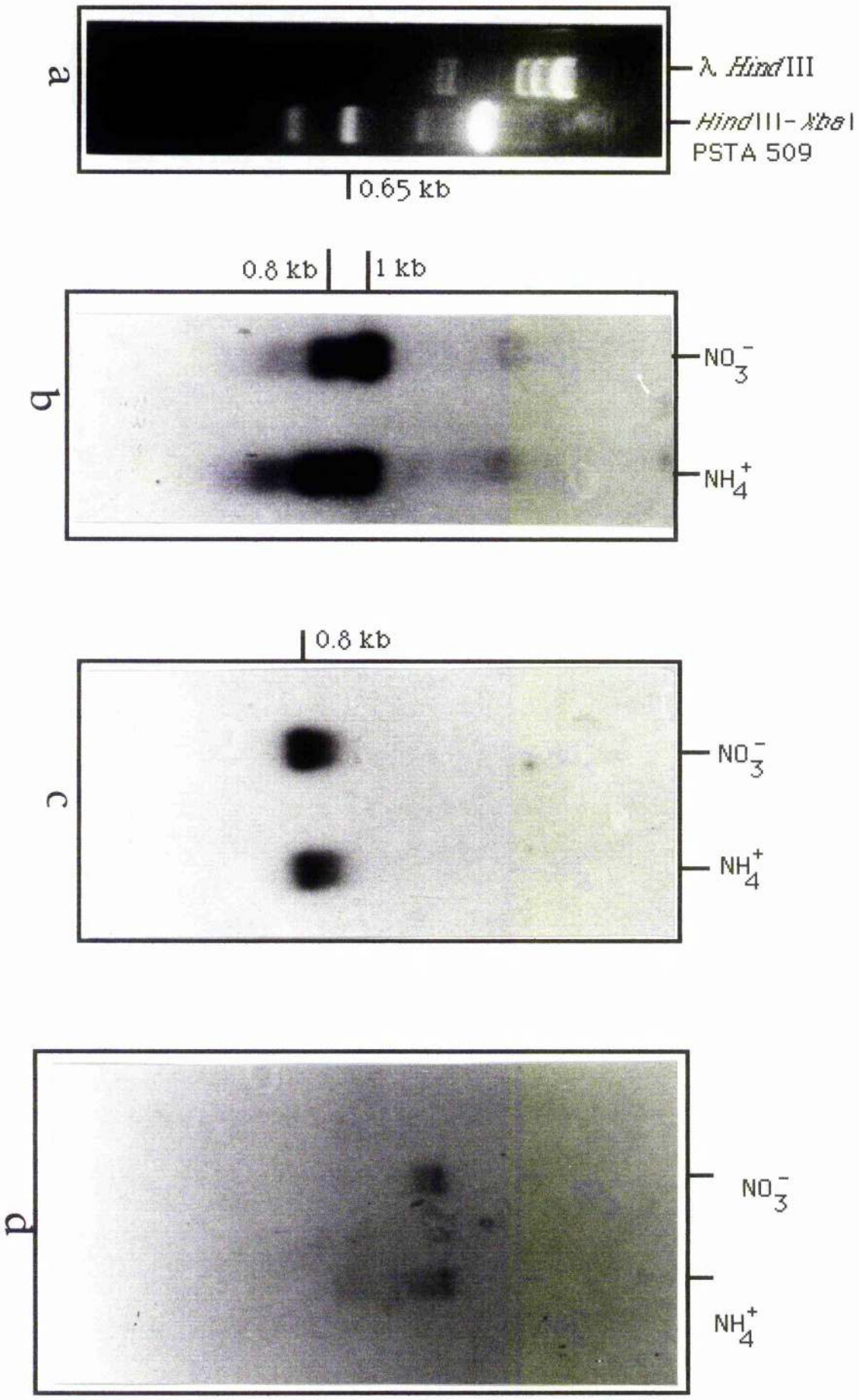
Messenger RNA was prepared from wild-type mycelia grown in minimal media with either ammonium (10 mM) or nitrate (10 mM) as the sole nitrogen source. In the first blotting experiment, subclone PSTA509 containing *cnxH* was used to probe mRNA filters. Two transcripts were produced in cells grown on either nitrogen source (Figure 6.18a). The size of these messages are estimated as 1 kb and 0.8 kb. In a second experiment, a 0.65 kb *Hind* III-*Xba* I fragment (Figure 6.18b) containing only the coding region of the PSTA509 subclone, was used to probe similar mRNA filters (Figure 6.18c). In this case only one signal was observed in both sets of cells suggesting an mRNA of 0.8 kb. In cells grown on nitrate the signal appeared stronger, than for growth on ammonium. However, an actin-specific probe (Figure 6.18d) indicates a single message when either nitrate or ammonium was used as the sole nitrogen source. On nitrate this message was slightly stronger than on ammonium indicating a higher total RNA content in the nitrate grown preparation. These results did not suggest an induction of *cnxH* by nitrate i.e. the protein might be translated independently on the nitrogen source or with little regulation at least at the transcriptional level.

6.7 Discussion.

The highest number of mutations were generally found in *cnxB* or *cnxF* loci. The possible explanation of these may be these mutant alleles are more sensitive to mutagenic treatments. The low

Figure 6.18. Northern Blot And The Regulation Of *cnxH* Expression.

Messenger RNA was prepared from wild-type mycelia grown with either nitrate (10 mM) or ammonium (10 mM) as the sole nitrogen source. (a) Subclone Psta509 digested with *Hin* dIII-*Xba* I. (b) The subclone Psta509 was used to probe mRNA filters. Two transcripts were seen in either of the cases. The size of the messages on nitrate were kb and kb (c) *Hin* dIII-*Xba* I fragment of subclone PSTA 509 was used to probe mRNA and a single message was seen on either nitrate or ammonium, the message size on nitrate was kb whilst on ammonium was kb. (d) In the actin probe also a single message was seen on either of the nitrogen sources, on nitrate the size of the message was kb whilst on ammonium it was kb.


a
λ. HindIII
HindIII-XbaI
PSTA 509
0.65 kb
0.8 kb
1 kb
b
NO3-
NH4+
0.8 kb
c
NO3-
NH4+
d
NO3-
NH4+

frequency of some other mutants such as in *cnxH* might arise as a result of these mutant alleles being more resistant to the mutagenic treatment than others. Another explanation is that, mutants in *cnxH* could be subject to active repair mechanisms more than other loci where such mechanisms can make the repair of the mutation very quickly.

Complementation analysis indicates that the highest percentage of *cnxB* mutants (approx 60%) arise as a result of NTG treatment of wild-type strains when glutamate was used. In contrast, when the same strains were treated spontaneously or with DEO approx 0% to 3% was seen. However, from the *gdh-niaD* strain SAA1040 50% *cnxB* mutant alleles generated after DEO mutagenesis as compared to 0% when SAA1032 strain was used. These findings would suggest that both the treatment and the strain used greatly influences the frequency of *cnxB* mutants generation i.e. the mutagen DEO was not suitable for generating *cnxB* mutants when used with wild-type strain (instead NTG was generating *cnxB* mutants with the wild-type). However, the DEO mutagen affected greatly the SAA1040 strain. When the wild-type strain was treated with DEO mutagen on uric acid media at 37°C was generating approximately 95% *cnxC* mutant alleles. Instead when mutants were selected at 25°C under the same treatment conditions, *cnxF* mutant alleles formed 50% of the analysed mutants. These results indicate that when only the selection temperature was changed a high frequency of mutant alleles representing two different genes was obtained.

This may suggest that the selection temperature influences the type of generated *cnx* mutants. However, after DEO treatment of all *gdh-niaD* strains no alleles of *cnxC*, *cnxE* or *cnxH* were obtained, which implicates again the influence of the treated strain on the frequency of generated mutants is significant. Moreover, after DEO treatment of strain SAA1032, no *cnxF* mutant alleles were obtained when ammonium was used as the sole nitrogen source, whilst, in the presence of the same nitrogen source (i.e. ammonium) 38% *cnxG* mutants was unearthed under the same mutagenic conditions. However, when the same strain (i.e. SAA1032 strain) and mutagen (i.e. DEO) were used with urea as sole nitrogen source 50% *cnxF* mutant alleles of the total analysed mutants was scored. In contrast, no *cnxG* mutants were obtained under such conditions. These findings would suggest that urea as a sole nitrogen source was influencing, positively, the frequency of *cnxF* mutant alleles (in this case approx 50% were obtained). Unexpectedly, when ammonium after DEO treatment served as sole nitrogen source with SAA1032 strain, the situation was very different. This nitrogen source affected positively the frequency of *cnxG* (i.e. approx 38%) and negatively that of *cnxF* mutant alleles (0%). These results would give a strong indication that the effect of the nitrogen source on the frequency of generated mutants. After NTG treatment of the wild-type strains, neither uric acid nor glutamate at 37°C were able to generate even a single *cnxE* or *cnxF* mutant. Such results suggests that the effect of either the nitrogen source, the selection temperature or even a combination of both factors on the rate of generated mutants.

The results obtained from *in vitro* examination of NR temperature stability studies indicated that the enzyme produced by two of the *cnxH* (i.e. *cnxH255* and *cnxH261*) mutants was more heat labile than that of the wild-type. This heat lability was approximately 40% reduction in the half-life as compared to the wild-type assayed under the same conditions. In addition *cnxA140* showed also a reduced enzyme activity as compared to wild-type and other *cnx* mutants but, such activity in *cnxA140* was approximately 3 times that of *cnxH* mutants. MacDonald and Cove (1974) have suggested that the *cnxH* gene might specify a small polypeptide which could be a structural component of nitrate reductase. The current work supports that proposal. In contrast, mutants having an enzyme with a half-life similar to that of the wild-type might specify an enzyme responsible for the synthesis of the molybdenum cofactor (MacDonald and Cove, 1974).

Furthermore, since no NR enzyme activity was observed at either the permissive or the non-permissive temperature in the non-conditional *cnx* mutants, this would suggest that such mutants have a point mutation at a crucial region of an essential protein (for example. in the active site of the enzyme) or a region involved in folding of the protein which is necessary for the function of the protein. However, this might not be the case with the thermo-sensitive mutants, that showed NR activity at the permissive but not at the non-permissive temperature. The activity of the enzyme in such mutants could be related to a point mutation in an area

responsible for the folding process of the protein. At the permissive temperature the protein may fold correctly, whilst at the selection temperature abnormal folding of the protein might occur. This change in protein configuration could cause loss of enzyme activity at the selection temperature whilst the enzyme active at the permissive temperature.

Furthermore, in some cases point mutations might cause small change in the protein and this would cause reduction of enzyme activity but not complete loss of this activity. In such cases temperature-conditional mutants might show a phenotype of weak growth at the selection temperature which should be much poorer than that of the wild-type strain. This kind of poor growth may be the case with the mutants that have not been included in the enzyme assay. However, the drastic change i.e. showing growth only at the permissive temperature) might not be explained by mutations at the active site of the enzyme but can be explained by change in protein configuration.

The *cnxH* gene was isolated by *A. nidulans* self-cloning transformations using the molecular services of the autonomously replicating plasmid pHELP. This cloning approach proved to be successful after other approaches such as chromosome walking had failed. Positive identity of the clone came from high transformation and complementation frequencies. The inferred *Aspergillus cnxH* protein sequence is similar and therefore the homologue of the

Escherichia. coli moaE product. This encodes an enzyme which is thought to carry out a catalytic step in molybdenum biosynthesis (Figure 1.4 and see introduction). Specifically the *cnxH* gene product by extension is involved in the regulation (i.e. converting the large subunit into active MPT) of the converting factor (MPT synthase) which converts the precursor into molybdopterin (MPT). This confirmed positive identity of the clone therefore, being shown to be involved in Mo-Co synthesis.

Sequencing of one temperature-sensitive and two temperature non-conditional *cnxH* mutants were carried out in an effort to shed light on *cnxH* structure / function relationships. Unfortunately both mutations in the non-conditional mutants generated stop codons which basically wrecked the *cnxH* protein and little or no information was provided except to confirm that all are *cnxH.* However, the mutation in *cnxH261* ^{ts} was found to be due to the insertion of glycine codon at position + 443 . This amino acid insertion leads to heat lability of the NR enzyme. More *cnx* mutants altered in the protein will be sequenced.

It may have been anticipated that *cnxH* expression levels might have been higher in nitrate grown cells, since nitrate reductase activity is higher (about 100-fold) in nitrate grown compared with ammonium grown cells with a similar difference at the NR mRNA level. In contrast, *cnxH* transcript levels are about the same under both conditions suggesting that little regulation of *cnxH*, at least at the transcriptional level, takes place.

Table 6.1. Genetical Analysis Of 456 *cnx* Mutants By Functional Complementation In Heterokaryons Grown On Nitrate As The Sole Source Of Nitrogen.

Treatment	Spontaneous		NTG					DEO						
Strain	G1and A220		G1 and A220					G1				SAA1040		
N-source	Glu	Pro	Glu		Pro	U.A		Glu		U.A		Pro	NH_4^+	Urea
Selection Temp	25°C	25°C	25°C	37°C	25°C	25°C	37°C	25°C	37°C	25°C	37°C	37°C	37°C	37°C
cnxA	0	4	18	0	8	1	2	0	0	0	0	0	0	5
cnxB	2	8	15	6	18	9	4	2	0	1	1	1	7	24
cnxC	0	1	2	1	7	0	2	0	1	3	19	0	0	2
cnxE	0	4	1	1	3	1	0	0	0	1	0	4	2	4
cnxF	0	9	23	0	16	9	5	1	0	6	0	1	3	2
cnxG	2	4	9	0	14	8	3	0	1	1	0	0	2	7
cnxH	0	1	3	2	3	2	2	0	0	1	0	0	0	3
Total no of analysed mutants	4	31	71	10	69	30	18	3	2	13	20	6	14	47
Total no of isolated mutants	4	94	128	14	178	32	18	3	2	22	22	6	21	836

Table 6.1. Continued.

Treatment	DEO						
Strain	1032			1023	1023b		
N-source	Pro	NH_4^+	Urea	Pro	Pro	NH_4^+	
Selection Temp	37°C	37°C	37°C	37°C	37°C	37°C	Total no of *cnx* in each group
cnxA	0	0	0	1	1	0	39
cnxB	4	6	0	2	11	8	130
cnxC	0	0	0	0	3	2	43
cnxE	5	4	2	1	10	11	54
cnxF	2	0	2	2	9	10	100
cnxG	0	6	0	1	4	5	68
cnxH	0	0	0	1	2	2	22
Total no of analysed mutants	11	16	4	8	37	41	0
Total no of isolated mutants	31	84	4	15	318	230	0

Table 6.2. Summary Of Temperature Conditional *cnx* Mutants.
(a) Cryso-Sensitive *cnx* Mutants

Mutant number	Complementation group	Thermo-sensitive	Cryo-sensitive	Gene designation	Other marker(s)	Strain number
97	*cnxB*		√	*cnxB97*^{cs}	*yA2, pyroA4*	GK.1
75	*cnxB*		√	*cnxB75*^{cs}	*yA2, pyroA4*	GK.2
1236	*cnxB*		√	*cnxB1236*^{cs}	*biA1*	GK.3
1277	*cnxB*		√	*cnxB1277*^{cs}	*biA1*	GK.4
418	*cnxC*		√	*cnxC418*^{cs}	*biA1*	GK.5
485	*cnxC*		√	*cnxC485*^{cs}	*biA1*	GK.6
399	*cnxC*		√	*cnxC399*^{cs}	*biA1*	GK.7
910	*cnxF*		√	*cnxF910*^{cs}	*biA1*	GK.8
482	*cnxF*		√	*cnxF482*^{cs}	*biA1*	GK.9

822	*cnxF*		√	*cnxF822*^{cs}	*biA1*	GK.10
140	*cnxA*	√		*cnxC140*^{ts}	*yA2, methH2*	GK.11
224	*cnxB*	*		*cnxB224*^{ts}	*yA2, methH2*	GK.12
232	*cnxC*	√		*cnxC232*^{ts}	*yA2, methH2*	GK.13
465	*cnxC*	*		*cnxC465*^{ts}	*yA2, methH2*	GK.14
246	*cnxE*	*		*cnxC246*^{ts}	*yA2, methH2*	GK.15
142	*cnxF*	√		*cnxC142*^{ts}	*yA2, methH2*	GK.16
384	*cnxF*	*		*cnxC384*^{ts}	*yA2, methH2*	GK.17
251	*cnxH*	√		*cnxC251*^{ts}	*yA2, methH2*	GK.18
255	*cnxH*	√		*cnxC255*^{ts}	*yA2, methH2*	GK.19
261	*cnxH*	√		*cnxC261*^{ts}	*yA2, methH2*	GK.20

* **(ts)** mutant growth phenotype with nitrate, but mutant at both temperatures(37° C,25° C) with hypoxanthine as sole nitrogen source. (√) ts or cs mutant growth mutant phenotype with nitrate and hypoxanthine as sole nitrogen source. All *cnx* mutants either ts or cs showed chlorate resistance at both temperatures.

Table 6.3. The Genetic Analysis Of *cnx* Mutants: A Summary.

Complementation Group / MutantType	*cnxA*	*cnxB*	*cnxC*	*cnxE*	*cnxF*	*cnxG*	*cnxH*
Non-Conditional	38	125	38	53	95	68	19
ts NO_3^-		1	1	1	1		
ts NO_3^-HY	1		1		1		3
cs NO_3^-HY		4	3		3		

Total number of chlorate resistant mutants isolated was 11807; of these *cnx* mutants were identified 2082; of the latter mutants 456 were genetically analysed. thermo-sensitive mutants 10; cryo-sensitive mutants 10.
ts NO_3^- : denotes thermo-sensitive on nitrate, but mutant at both temperatures (37°C,25°C) on hypoxanthine.
ts NO_3^- HY: denotes thermo-sensitive mutant on both nitrate and hypoxanthine.
cs NO_3^- HY: denotes cryo-sensitive mutant on both nitrate and hypoxanthine.

Table 6.4. Isolation Of Temperature Conditional *cnx* Mutants From This Study And Previous Published Work (MacDonald and Cove 1974)

* Thermo-sensitive or cryo-sensitive mutant phenotype on nitrate, but mutant at both temperatures (37°C, 25°C) on hypoxanthine.

Thermo-sensitive or cryo-sensitive mutant phenotype on nitrate and hypoxanthine.

• Arst *et. al.*, (1982) isolated one *cnx*^{cs} (*cnxC20*) on nitrate or hypoxanthine.

This study :

◊ The above *cnx* mutants were isolated from both wild-type and *gdh-niaD* transformant strains (SAA1040, 1032, 1023a, 1023b) as described in materials and methods .

In this study: (1) number of chlorate resistant mutants of wild-type origin 5274; of these 537 *cnx* mutants ; (2) number of chlorate resistant mutants of *gdh-niaD* transformants 6533; of these 1545 *cnx* mutants.

Cove's(1974) study:

2246 *cnx* mutants were analysed; 14 were identified as thermo-sensitive mutants. No attempt

was made at isolating cryo-sensitive mutants.

Gene	This Study				Previous Study	
	ts		cs		ts	
	NO_3^-*	NO_3^- HY•	NO_3^-	NO_3^- HY	NO_3^-	NO_3^- HY
cnxA	0	1	0	0	0	0
cnxB	1	0	0	4	0	0
cnxC	1	1	0	3	0	0
cnxE	1	0	0	0	0	4
cnxF	1	1	0	3	0	7
cnxG	0	0	0	0	0	0
cnxH	0	3	0	0	3	0

Table 6.5 Wild-Type DNA Partail *Sau* 3A And argB Genomic Bank Were Used Along With Helper Plasmids To Transform Either The Double (GH5.4) Mutant *argB cnxH4* Or The Single (063) Mutant *cnxH4*. Each confirmed *Aspergillus* transformant which its DNA was used to transform *E. coli* is designated by certain lettre. Total number of *E. coli* transformants obtained from each *Aspergiluus* transformant are presented in opposite to that transformant. For example pool 2 originally from 3 *Aspergillus* transformants designated a, b and c, transformant a yielded one *E. coli* transformants, b yielded 7 *E. coli* and c yielded 2 *E. coli* transformants.

Table 6.5 Transformation Frequencies Obtained With *cnxH* Single And Double Mutants.

Donor DNA	Transformed strain		Confirmed transformants		Transformants used for *E.coli* transformation		*E. coli* transformants		Pool number
	GH5.4	063	GH5.4	063	GH5.4	063	GH5.4	063	
5 μg argB genomic bank + 15 μg pHELP/*Bam* HI	173	16	5	1	1	1	44	2	1 for 063 3 for GH5.4
5 μg partial *Sau* 3A genomic DNA + 15 μg pHELP		69		3		3 a b c		1 7 2	2
5 μg argB genomic bank	149		10		2 d e		3 8		4
5 μg partial *Sau* 3A genomic + 15 μg pDHG25	26		2		2 f g		14 7		5
5 μg partial *Sau* 3A genomic + 15 μg pDHG25 (another preparation)	37		9		1		11		6
5 μg partial *Sau* 3A genomic + 15 μg pHELP (another preparation)		5		4		4 h i j k		4 1 2 1	7

Table 6.6 a And b. Total Number Of *E. coli* Transformants Representing Each Pool Are Shown In Table 6.5. plasmid DNA pools of wild-type partial *Sau*3A digest and pHELP were divided into subpools, where all *E. coli* transformants from a single *Aspergillus* transformant were representing certain subpool. Subpools were transformed with *cnxH4* strain and transformation frequencies obtained are shown in the Table.

Table 6.6a Retransformation Of *cnxH4* Frequency Obtained With DNA Pools.

Pool number	1	2	3	4	5	6	7
Number of transformants	51	248	493	381	266	112	310

Table 6.6b Retransformation Frequencies Of *cnxH4* Obtained With Subpools Of Wild-Type *Sau*3A Digested And pHELP/*Bam*HI Digested.

Pool number	2			7			
Subpool	a	b	c	h	i	j	k
E. coli transformants	1	7	2	4	1	2	1
Aspergillus transformants (4 independent experiments)	30	9	0	17	0	0	0
	4	27	0	16	0	0	0
	118	168	0	197	0	0	0
	143	163	0	8	0	0	0

Table 6.7. Transformation Frequencies Of Mutant *cnxH3*.

Donor DNA	*Aspergillus* transformants	Confirmed transformants	Transformed to *E.coli*	*E. coli* transformants
2 μg partial *Sau* 3A genomic + 5 μg pHELP/*Bam*HI	1	1	1	14
1 μg argB genomic bank + 5 μg pHELP/ uncut	7	2	1	0
10 μg argB genomic bank + 5 μg pHELP/uncut	24	14	1	0
5 μg argB genomic bank	2	2		
5 μg pHELP/*Bam* HI control	0	0		

The *Aspergillus* transformants obtained were subcultured on nitrate (selective media) and after the observed unstable growth such transformants were subcultured onto complete media (non-selective media) then subcultured onto nitrate again. Subculturing from non-selective to selective lead to loss of ability of these transformants to grow due to loss of complementing plasmid on the non-selective media.

Table 6.8. Retransformation Frequency Of Aspergillus *cnxH* Using Rescued Plasmids. From a total of 14 *E. coli* transformants, 6 colonies were purified on Luria containing ampicillin and 2 colonies from each size range were used for plasmid DNA preparation. Four plasmids (p5.2, p5.4, p5.5 and p5.6) were rescued in *E. coli* and re-transformed into *cnxH* mutations.

Table 6.8. Retransformation Frequency Of *Aspergillus cnxH* Using Rescued Plasmids.

Transformed Strain	Doner DNA	Number of transformants.
∝ 56 *biA1 cnxH3*	3 μg p5.2 + 2 μg pHELP/*Bam*HI	0
	3 μg p5.4 + 2 μg pHELP/*Bam*HI	~1000
	3 μg p5.5 + 2 μg pHELP/*Bam*HI	~1000
	3 μg p5.6 + 2 μg pHELP/*Bam*HI	~1000
	2 μg pHELP/*Bam*HI	0
G338 *pantoC3 Sc12 cnxH3*	3 μg p5.5 + 2 μg pHELP/*Bam*HI	~1000
	3 μg p5.6 + 2 μg pHELP/*Bam*HI	~1000
	3 μg p5.6 + SDW	~1000
	2 μg pHELP/*Bam*HI	0
G338 *pantoC3 Sc12 cnxH3*	3 μg p5.6 + 2 μg pHELP/*Bam*HI	~2000
	2 μg pHELP/*Bam*HI	0

Table 6.9. Retransformation Frequency Of Different Digests Of The Rescued Plasmid p5.6.

Transformed Strain	Donor DNA	Number of transformants.
cnxH4	4 μg p5.6/*Eco* RI +1 μg pHELP/*Bam*HI	459
	4 μg p5.6/*Bam*HI + 1 μg pHELP/*Bam*HI	24
	4 μg p5.6/*Hind*III + 1 μg pHELP/*Bam*HI	480
	4 μg p5.6 + 2 μg pHELP/*Bam*HI	~1500
	4 μg pHELP/*Bam*HI	0

The rescued plasmid p5.6 digested with three endonucleases (*Eco* RI, *Bam* HI and *Hin* dIII) was transformed into *cnxH4* strain. Both *Hin* dIII and *Eco* RI digests were transforming *cnxH4* mutation at high frequency.

Table 6.10. Retransformation Frequency Of Recombinant argB Clones Into *Aspergillus* Strain *cnxH4*. The clone p5.6 was cleaved with *Eco* RI and 10 bands were isolated. Of the ten cleaved bands only one (band number 9) complemented *cnxH4* mutant. The complemented band was ligated to pUC18 and used as probe for hybridisation with argB genomic bank. Positive hybridising argB clones were transformed into *cnxH4* and the transformation frequencies obtained are shown in the Table.

Transformed Strain	Donor DNA	Number of transformants.
cnxH4	1 μg p5.6 +1 μg pHELP/*Bam*HI	~2000
	4 μg argB 3/3 + 1 μg pHELP/*Bam*HI	~1000
	4 μg argB 4/2 + 1 μg pHELP/*Bam*HI	100
	4 μg argB 5/1 + 1 μg pHELP/*Bam*HI	~1000
	4 μg argB 5/2 + 1 μg pHELP/*Bam*HI	10
	4 μg pHELP/*Bam*HI	0

Table 6.11. Retransformation Frequency Of *cnxH4* With Different Endonuclease Digests Of The argB Clones (argB5.1 And argB3.3). Two argB recombinant clones (i.e. argB3.3 and argB5.1) that showed high transformation frequency were digested with *Eco* RI. DNA was prepared from the cleaved fragments in both clones and transformed with *cnxH4* mutant strain. The results indicate that *Eco* RI fragment 1.9 kb of argB 5.1 clone gave the highest transformation frequency.

Transformed mutant	Donor DNA	Number of transformants.
cnxH4	4 μg p5.1 +1 μg pHELP/*Bam*HI	~500
	4 μg argB5.1/*EcoR* I 1.9 kb + 1 μg pHELP/*Bam*HI	~300
	4 μg argB5.1/*EcoR* I 5 kb + 1 μg pHELP/*Bam*HI	30
	4 μg argB3.3 + 1 μg pHELP/*Bam*HI	~500
	4 μg argB3.3/*EcoR* I 1.7 kb + 1 μg pHELP/*Bam*HI	3
	4 μg argB3.3/*EcoR* I 1.9 kb + 1 μg pHELP/*Bam*HI	17
	4 μg argB3.3/*EcoR* I 3 kb + 1 μg pHELP/*Bam*HI	200
	4 μg argB3.3/*EcoR* I 5 kb + 1 μg pHELP/*Bam*HI	5
	4 μg pHELP/*Bam*HI	0

Table 6.12. Retransformation Of *cnxH4* With Three argB Subclones Taken From The Original Clone argB5.1. The complementing *Eco* RI fragment 1.9 kb of argB5.1 clone was ligated to pUC18, *Eco* RI digested DNA indicate the presence of three subclones designated H1, H4 and H8. DNA prepared from these subclones was transformed with *cnxH4* along with the original clone as indicated in the Table.

Host	Donor DNA	Number of transformants.
cnxH4	1 μg argB 5/1 + 1 μg pHELP/*Bam*HI	~400
	1 μg H1 +1 μg pHELP/*Bam*HI	~200
	4 μg H4 + 1 μg pHELP/*Bam*HI	400
	4 μg H8 + 1 μg pHELP/*Bam*HI	~1000
	1 μg p5.1/*Eco* RI 1.9 bp + 1 μg pHELP/*Bam*HI	~300
	4 μg pHELP/*Bam*HI	0

CHAPTER SEVEN

ATTEMPTS AT CLONING *Arabidopsis thaliana cnx* GENES.

Extensive experiments by a visiting research worker to St. Andrews (Jorg Neider, Technical University of Braunsweg, Germany) provided evidence that genomic *Arabidopsis* DNA could be cloned in *Aspergillus nidulans* on the basis of the complementation of *A. nidulans* mutants. Neider used the pHELP system to introduce *Arabidopsis* DNA into *Aspergillus* using *A. nidulans cnxE* mutants to generate equivalent *Arabidopsis cnx* clones. Unfortunately after investigation these claims could not be confirmed.

During the period of confirmation I attempted to isolate *Arabidopsis* clones in an almost identical manner. since I am intrinsically interested, in addition, to fungi in plant molecular biology, I decided, with the permision of the supervisor, to carry out the introduction of *Arabidopsis thaliana* DNA into *Aspergillus nidulans* to generate new equivelent *Arabidopsis cnx* clones. Partial *Sau* 3A *Arabidopsis* DNA along with the vector pHELP cleaved with *Bam* HI was transformed with the *Aspergillus cnxC3*, *cnxF7* and *cnxH4* mutant strains. This self-cloning technique was used for over 6 months with an average of 3 to 4 transformation experiments per week. During this period such technique has been tried also by a collaborator (Mr. Eddie Campbell). Not surprisingly, no success was gained in any

of the experiments. When Dr. Kinghorn found that such results were not correct and since these claims could not be confirmed, we were asked to stop this research section.

CHAPTER EIGHT.

DISCUSSION, CONCLUSIONS AND FUTURE WORK.

During the course of this work a relatively large number (i.e. over 11,000) of mutants were isolated on the basis of chlorate resistance. 425 mutants, phenotypically resembled *crn* (chlorate resistant, nitrate utilising) nitrate transport mutants whilst 2082 *cnx* (molybdenum cofactor for nitrate reductase and xanthine dehydrogenase, (now called purine hydroxylase): some isolates were purified and used in genetic, biochemical and molecular studies.

The *crn* mutants were found to map in four distinct unlinked loci *crnA*, *crnB*, *crnC* or *crnD*. Before this current study only the *crnA* gene had been recognised.

The protein product of the *A. nidulans crnA* gene i.e. CRNA had been already implicated in nitrate transport (Brownlee and Arst, 1983; Unkles *et. al.*, 1991) for the following reasons. First, *crnA* mutants showed low nitrate uptake levels as compared to wild-type. Secondly, the CRNA protein showed the hallmarks of a transport system which are : (1) Northern blot analysis indicate that crnA expression is induced by nitrate and repressed by ammonium similar to other nitrate assimilation genes such as niaD and niiA. (2) Its hydropathy profile shows 12 hydrophobic membrane spanning domains. (3) crnA1 mutation has reduced nitrate uptake rates in younger cells approximately 5-fold. During this study a number of

further *crn* mutants have been considered to be *crnA* alleles since the recombination is very low (i.e. no recombinant progeny, chlorate sensitive, in the 50 progeny analysed) in pair-wise crosses with *crnA1*. Some of these were sequenced at the DNA level. Surprisingly with some of these proposed *crnA* mutants no nucleotide changes were observed in sequencing experiments in which both nucleotide strands had been determined. Although, there remains the possibility of a sequence technical problem, it would appear from these results that there are one or more *crn* genes adjacent to *crnA* and distal to *niiA -niaD* genes (i.e. extending outwards from *crnA* on the other side of *crnA* as *niiA-niaD*). *crnA* could be a locus consisting of several genes or these mutants could be promoter ones. This question may be resolved by molecular 'walking' along to these genes, isolating fragments and sequencing these. Their protein sequence should give as clues as to their function.

There are three hitherto unidentified *crn* loci *crnB*, *crnC* and *crnD* which are unlinked to each other, by analogy with *crnA*, there may be several genes at *crnB* or *crnC* loci (*crnD* is only represented by one mutant. The defect(s) in the *crnB*, *crnC* and *crnD* mutants are unclear. Certain of these mutants have reduced nitrate uptake capacity and by analogy with the *crnA* mutants, may specify individual components for a complex nitrate uptake system. Alternatively, the other *crn* genes may encode distinct and individual nitrate transporters, independent of the *crnA* transporter

and each other. Another distinct possibility is that the other *crn* products are involved in the passage of the CRNA protein to the membrane i.e. in trafficking through the Golgi body by acting for example, as molecular chaperones or involved in the golgi body. Molecular cloning of *crnB*, *crnC* and *crnD* genes and any further *crn* genes (i.e. adjacent to *crnA* as discussed above) and determination of their DNA sequences should provide evidence which may clarify these uncertainties. In this regard, such approaches has already successfully provided strong evidence that CRNA is clearly a transporter (Unkles *et. al.*, 1991).

A number of mutants in the *crnA* gene were sequenced to determine the amino acid defect or alteration in the protein in order to give us information on CRNA structure / function relationships. In this respect the original *crnA1* mutant as well as the *crnA1009* and *crnA1087* were analysed. As the *crnA1* mutation basically results in the complete loss of CRNA protein (from sequence results) this means that the remaining nitrate uptake capacity in this mutant is due to another system(s) being present-thus showing the presence of more than one system. *crnA1087* is a glycine to glutamate change in the transmembrane domain seven (Figure 1.2) Clearly this domain is important for nitrate transport since such a relatively small disturbance results in phenotypic change. Unfortunately the mutant *crnA1009* is less informative involving the disruption of the end of the protein. In general terms more detailed information on the topology of *crnA* will eventuate with the sequence determination

of many more *crnA* mutants isolated during the course of this thesis. Further interesting work could include the use of the green reporter protein (GRP) also called green fluorescent protein (GFP) which has been isolated from the squid and it flashs when exposed to fluorescent light. This protein has been used mainly in higher plants as a reporter gene when ligated to the promotor region of the gene of interest then transformed to Agrobacterium to manipulate the plasmid then transformed to the host for the purpose of localisation and distribution of the protein in cells. In *Aspergillus* this reporter might permit (after splicing into the C-terminal of the CRNA protein) Following the transit of CRNA through the cells secretory systems. Additionally, the location of CRNA in younger and older cells may be established. It might be that CRNA is only produced at the hyphal tips for instance.

In summary, we know the function of *crnA* and we have many mutants within the *crnA* gene which should give us insights into CRNA topology but we have no idea what roles the other genes play. This awaits another series of molecular experiments.

Mutations within *crnA*, *crnB*, *crnC* and *crnD* show resistance to chlorate but have an interesting additional phenotype; This allowed the crossing experiments that identified the additional *crn* genes sensitivity to caesium with nitrate as the sole nitrogen source (i.e. hypersensitive in comparison with the resistance wild-type). This "extra "phenotype is most likely part or the same as the chlorate resistant mutation in the *crnA* gene (or *crnB*, *crnC*, or

crnD) or is very tightly linked to *crnA*. More than ~ 400 progeny that were analysed in the outcrosses done during this study would have to be analysed to differentiate between the two possibilities. It is likely that both phenotypes are part of the same mutation, as (1) all *crnA* *crnB*, *crnC* and *crnD* mutants have this phenotype. (2). It is very unlikely that every mutant would have this 'double' phenotype. In this respect, double mutants are rare in any case, especially during spontaneous mutagenesis. It was found true that *crnB*, *crnC* and *crnD* mutants also have the double closely linked phenotype of chlorate resistance and caesium sensitivity. It is not clear what the molecular bases of this is but presumably caesium interferes with nitrate uptake and the cells are starved of nitrogen under this growth regime.

Two further genes were suggested during the course of the genetic studies carried out on *crn* mutants. First, *chlA* which is chlorate resistant, but caesium resistant (unlike *crn* mutants which are caesium sensitive as discussed above). Second, caesium sensitive, chlorate sensitive (again in contrast to *crn* mutants) were revealed and designated *cesA*. Mutants in *chlA* or mutants in *cesA* are unlinked to *crn* genes and to each other. Curiously these additional mutations were only found in *crnA*, *crnB* and *crnC* strains but not (as discussed above) in *crnD*. Unfortunately, no uptake data is available for these mutants in purified strains. More uptake work needs to be done as well as their molecular gene isolation to determine their roles, if any, in nitrate transport.

The results show that it is likely that there is an active nitrite uptake system in *A. nidulans* although it is not clear unfortunately if this is specific for nitrite or is a bispecific, sharing with nitrate. Nitrite uptake activity is repressed by ammonium in common with a large number of *A. nidulans* systems involved in nitrogen metabolism and induced by nitrate. Mutants are hypersensitive to nitrite (i.e. *meaB6*, *niiC628* and *tamA105*)which showed increase nitrite uptake perhaps not expectedly. It is not clear what the function of these genes are, perhaps regulatory as Hynes (Davis *et. al.*, 1996) and Caddick (Polley and Caddick, 1996) have suggested for *tamA* and *meaB* respectively. No specific nitrite transport defective mutants have been isolated as no mutant selection system is available.

Complementation analysis of the *cnx* mutants described 5 loci as before *cnxA*, *cnxB*, *cnxC*, *cnxE*, *cnxF*, *cnxG* and *cnxH*, with *cnxABC* being a complex locus with three complementation groups. Therefore, the results are similar to that of Cove (1979 and references therein). However, an additional but novel approach was used in this study in the hope that further *cnx* genes might be detected. In this respect the wild-type promotor of nitrate reductase was replaced by a glutamate dehydrogenase promotor (*gdhA* or *gdhB*, see introduction). This promotor is not controlled by nitrogen or ammonium repression. Therefore, transformants containing this gene hybrid were not protected by ammonium on chlorate containing media. These permitted unusual mutant selection

conditions to be used i.e. chlorate resistance in the presence of ammonium. A large number of *cnx* mutants were isolated under these conditions but unfortunately all fell into the existing *cnx* groups.

A further aim was to generate temperature sensitive *cnx* mutants which could be sequenced at a latter date (as Cove's original conditional mutants were lost or not made available to us). Temperature-sensitive *cnxH255* and *cnxH261* mutants isolated in this study had thermolabile nitrate reductase showing that it encodes a peptide which is part of NR enzyme. For *cnxA140* the activity of the enzyme was lower than that of the wild-type but three times that of *cnxH mutants*. In contrast, temperature-sensitive mutants in other *cnx* loci (i.e. *cnxC* and *cnxF*) showed similar NR thermostability to the wild-type NR enzyme. This indicates that such *cnx* genes are likely to be involved in molybdenum cofactor biosynthesis by way of activating enzymes rather than being structurally involved in the NR protein. These temperature-sensitive mutants will be helpful to evaluate structure / function relationships. In this regard, all the *cnx* genes have been isolated and at least partially sequenced in Dr. Kinghorn's lab, university of St. Andrews.

The *cnxH* gene was isolated by self-cloning technology, sequenced at the DNA level and the inferred protein found to have high similarity with the *E. coli* MOAE protein, the product of the *moaE* gene. This gene play an important role in the synthesis of

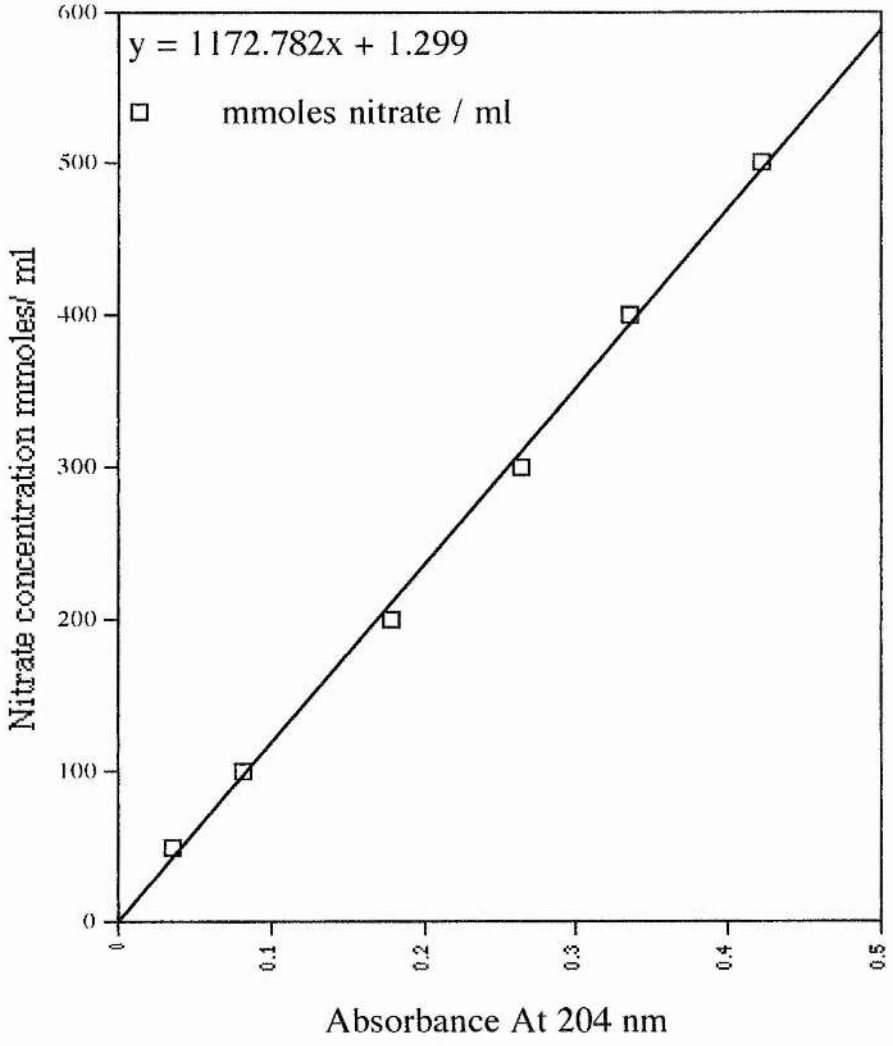
molybdopterin from the precursor Z (Figure 1.4) i.e. *cnxH* regulates MPT synthase. Two non-conditional *cnxH* mutants were sequenced; *cnxH427* and *cnxH911*. Both mutants generated stop codons and no information concerning structure / function relationships was provided. The two *cnxH* temperature-sensitive mutants *cnxH255* and *cnxH261* with much reduced half-life nitrate reductase should provide structural information. The *cnxH261* mutation was sequenced and found to have a GGG insertion resulting in the addition of glycine between residue positions 148 and 149 of the inferred amino acid sequence. This possibly results in a conformational change in the product of the *cnxH* gene. This protein product must as well as its *moaE* equivalent function i.e. as part of the converting factor of precursor Z be closely associated with the nitrate reductase molecule as it results in labile nitrate reductase (This thesis, MacDonald and Cove, 1974) it would seem therefore that there may be protein:protein interaction between NR and *cnxH*. In other words, this inserted amino acid lead to heat lability of the NR enzyme i.e. the half-life of the mutant enzyme was reduced approximately 40% as compared to the wild-type enzyme. It would appear that from the temperature sensitive studies that the *cnxH* (i.e. *moaE*) product is structural part of nitrate reductase. More specifically, although the NR enzyme is encoded by the structural gene *niaD*, there are other genes necessary for the nitrate assimilation pathway such as *cnx* genes. Only temperature-sensitive mutants in the *niaD* gene could probably affect the enzyme activity i.e. made the enzyme more heat labile. However, temperature-sensitive mutants in the *cnx* genes either would have wild-type

enzyme activity (at the selection temperature) or no activity (at the selection temperature). More clearly, temperature-sensitive mutants in *cnxC* or *cnxF* loci (isolated in this study), *cnxE* or *cnxF* again isolated previously (MacDonald and Cove, 1974) showed approximately wild-type enzyme activity at the non-selection temperature. In contrast, temperature sensitive mutants in *niaD* gene (MacDonald and, Cove,1974) or in *cnxH* mutants isolated in this study and in previous study showed much reduced half-life of the enzyme NR. This reduction in the half-life could be possibly related to the insertion of the amino acid glycine (in the *cnxH261* ^{ts}) which may have changed the configuration of the protein and lead to this heat liability of the enzyme. Since only *cnxH* temperature-sensitive mutants in addition to *niaD*, have reduced the enzyme activity these results would suggest that *cnxH* gene is specifying a structural component of the enzyme NR.

cnxH transcript levels are the same under both conditions, suggesting that no or little regulation of this gene, at least at the transcriptional level, takes place.

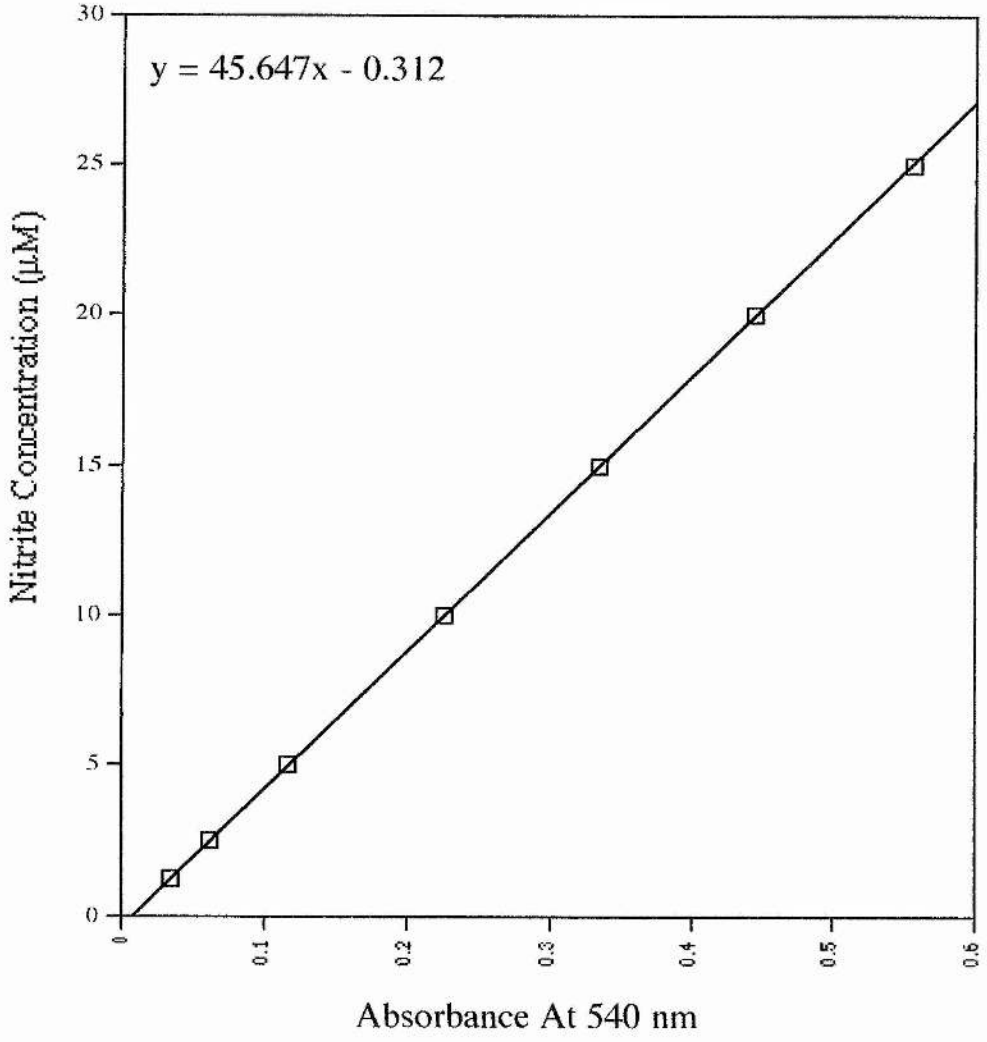
Appendices

Appendix 1



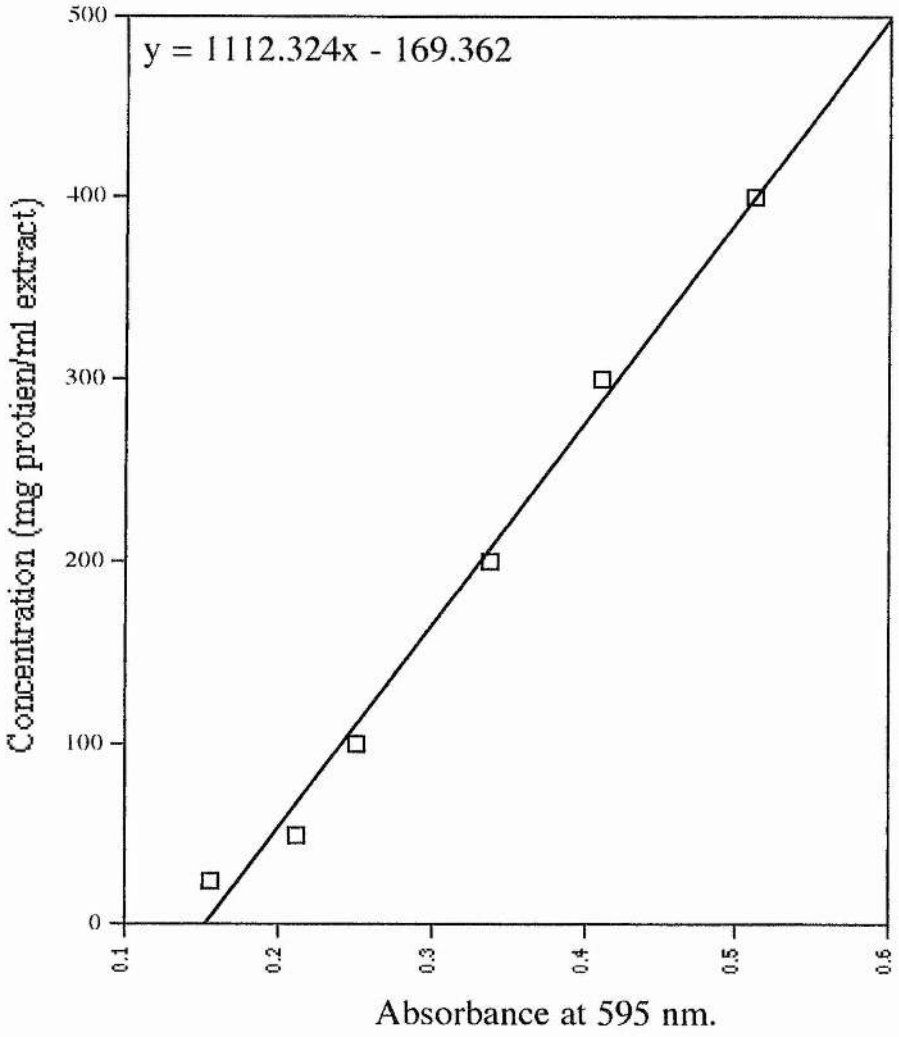
Nitrate standard calibration plot

Appendix 2



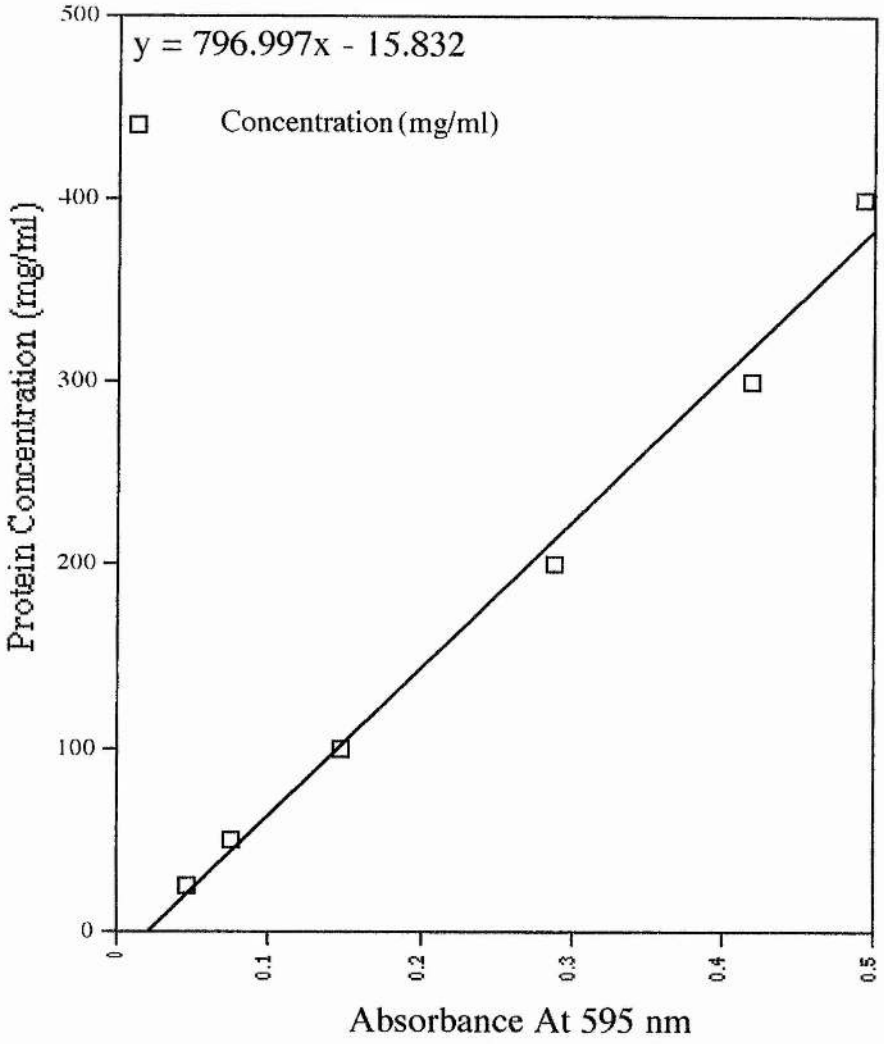
Nitrite standard calibration plot.

Appendix 3



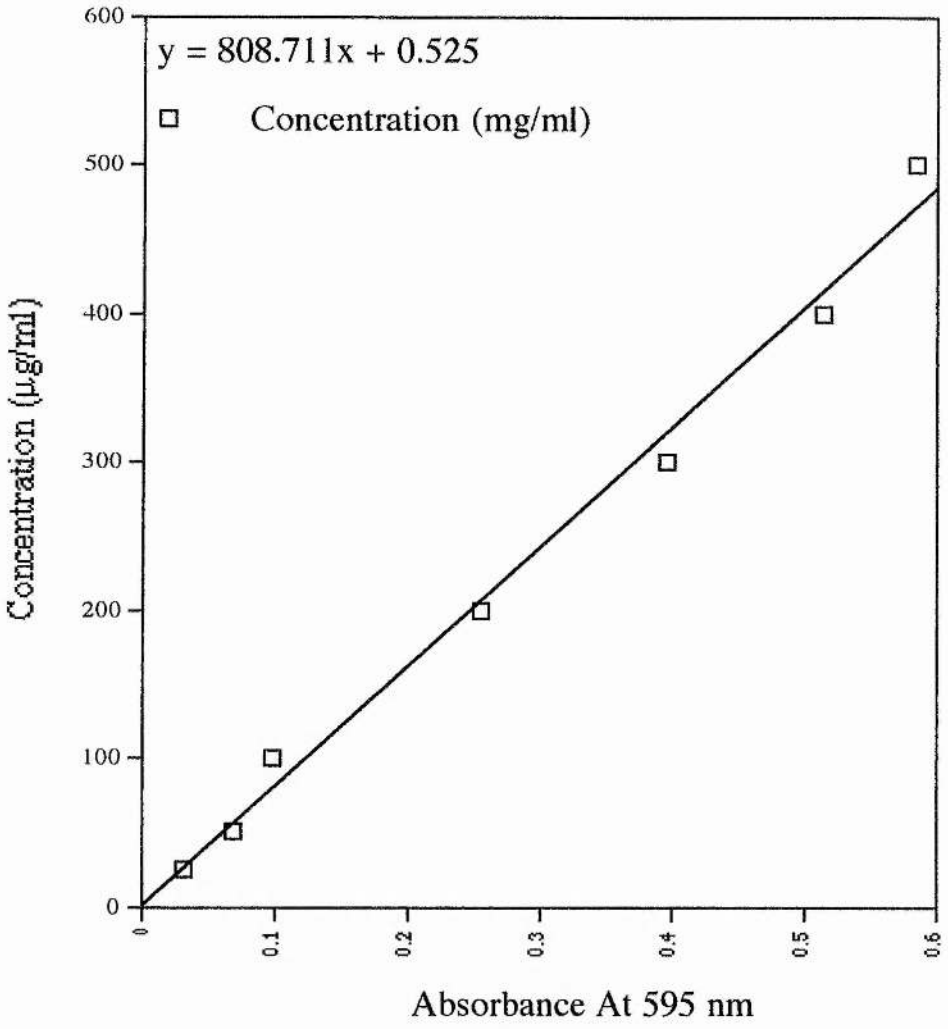
Representative protein standard calibration plot for nitrate reductase activity in *crn* mutants. The standard calibration plot was drawn by using the CA-cricket graph III programme designed for Apple Macintosh computers. The amount of soluble protein in the extracts was estimated by using the Bio-Rad protein standard II (lypholised BSA). protein content was estimated by measuring the absorbance at 595 nm (water was used to zero the spectrophotometer).

Appendix 4



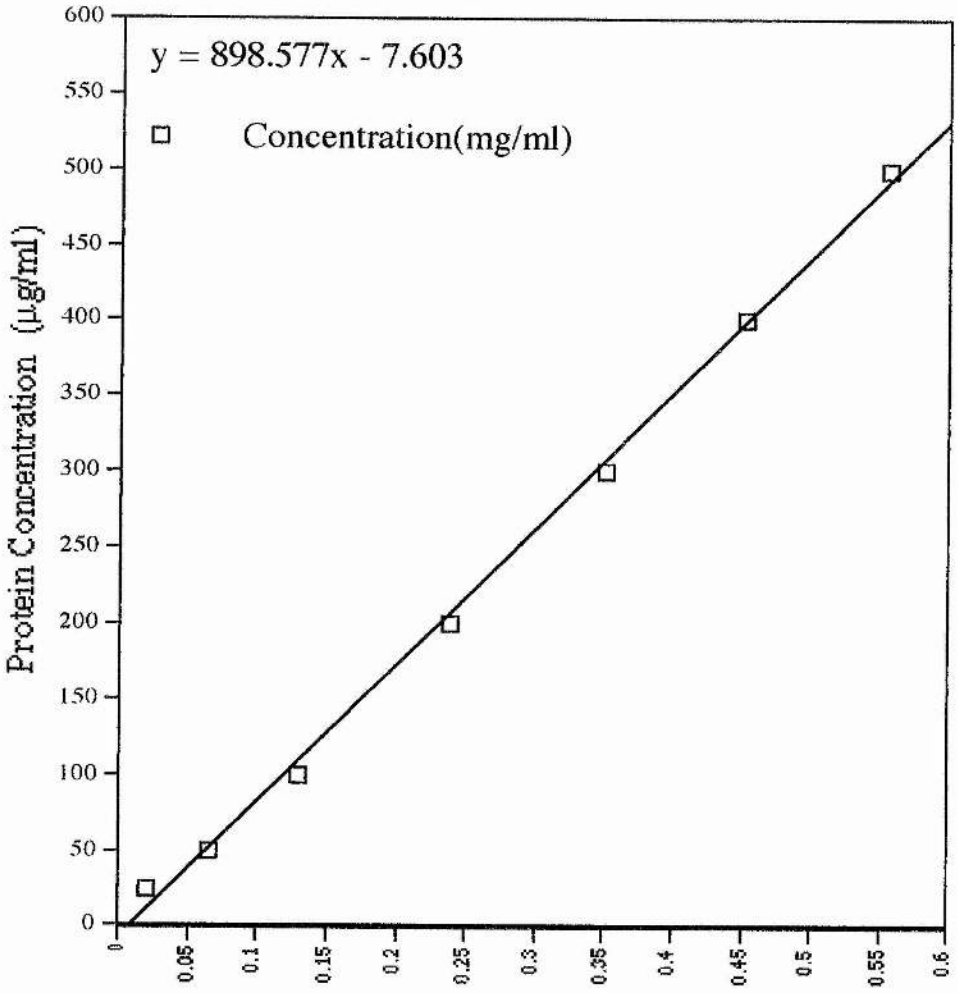
Representative protein standard calibration plot for nitrate reductase activity in wild-type and *niaD* transformants. The standard calibration plot was drawn using CA-cricket graph III programme designed for Apple Macintosh computer. The amount of soluble protein in the extracts was estimated by using the Bio-Rad protein standard II (lypholised BSA). Protein content was estimated by measuring the absorbance at 595 nm (water was used to zero the spectrophotometer).

Appendix 5



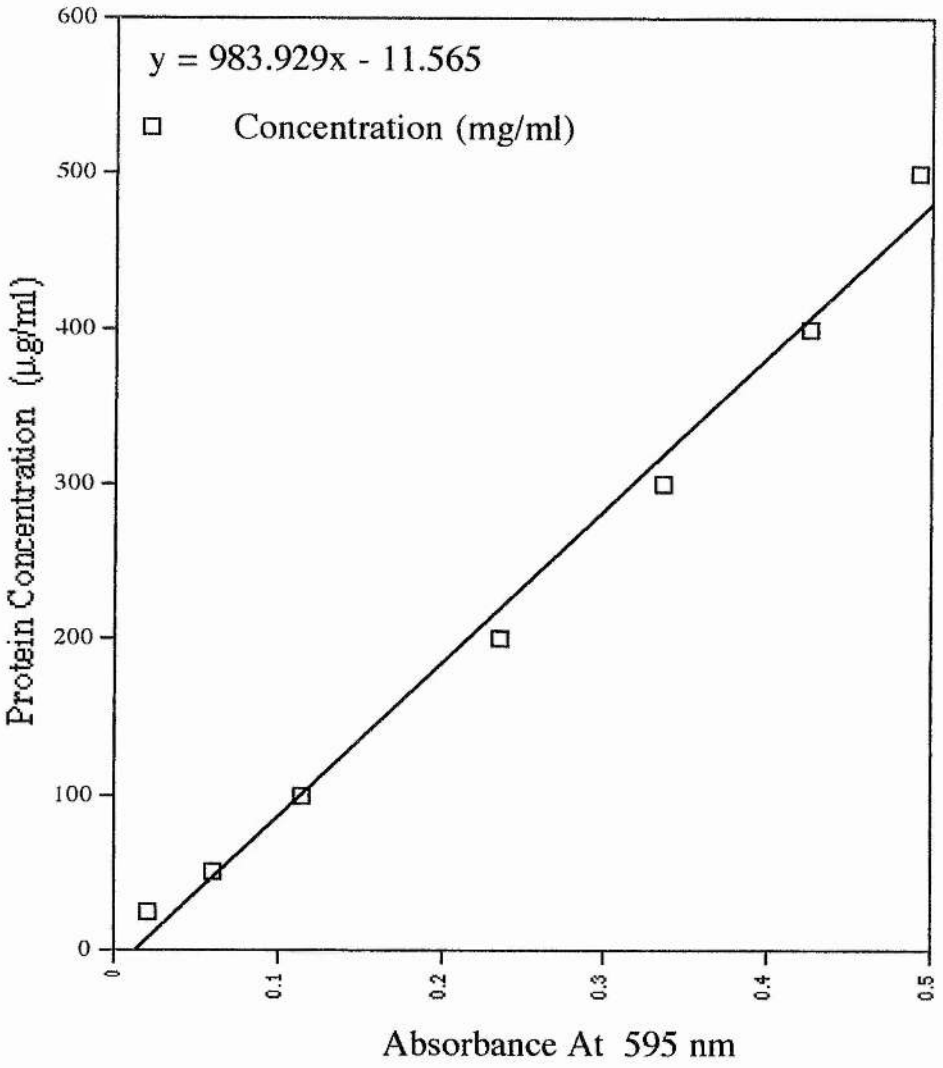
Representative protein standard calibration plot for nitrate reductase activity in wild-type and the temperature-sensitive *cnxA140* mutant. The standard calibration plot was drawn using CA-cricket graph III programme designed for Apple Macintosh computer. The amount of soluble protein in the extracts was estimated by using the Bio-Rad protein standard II (lypholised BSA). Protein content was estimated by measuring the absorbance at 595 nm (water was used to zero the spectrophotometer).

Appendix 6



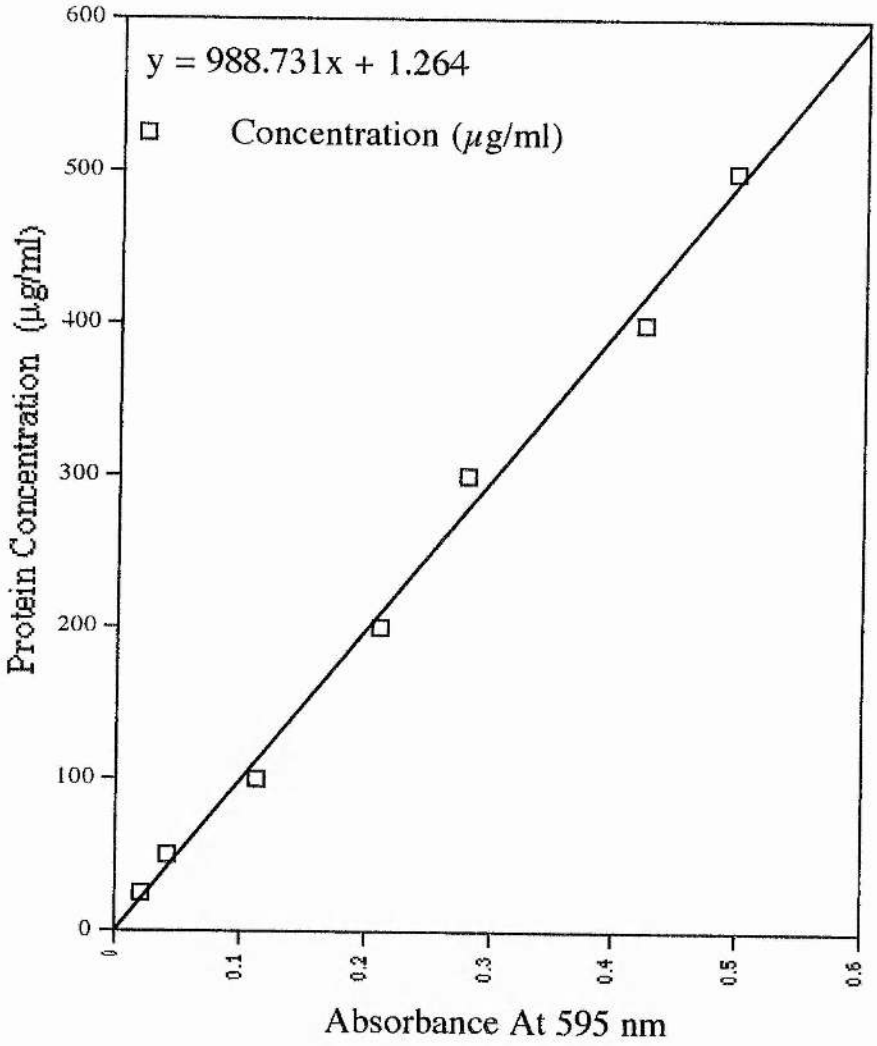
Representative protein standard calibration plot for nitrate reductase activity in wild-type and the temperature-sensitive *cnxC (cnxC232* and *cnxC 465)* mutants. The standard calibration plot was drawn using CA-cricket graph III programme designed for Apple Macintosh computer. The amount of soluble protein in the extracts was estimated by using the Bio-Rad protein standard II (lypholised BSA). Protein content was estimated by measuring the absorbance at 595 nm (water was used to zero the spectrophotometer).

Appendix 7



Representative protein standard calibration plot for nitrate reductase activity in wild-type and the temperature-sensitive *cnxF (cnxF142* and *cnxF384)* mutants. The standard calibration plot was drawn using CA-cricket graph III programme designed for Apple Macintosh computer. The amount of soluble protein in the extracts was estimated by using the Bio-Rad protein standard II (lypholised BSA). Protein content was estimated by measuring the absorbance at 595 nm (water was used to zero the spectrophotometer).

Appendix 8



Representative protein standard calibration plot for nitrate reductase activity in wild-type and temperature-sensitive *cnxH (cnxH251, cnxH255* and *cnxH261)* mutants. The standard calibration plot was drawn using CA-cricket graph III programme designed for Apple Macintosh computer. The amount of soluble protein in the extracts was estimated by using the Bio-Rad protein standard II (lypholised BSA). Protein content was estimated by measuring the absorbance at 595 nm (water was used to zero the spectrophotometer).

Bibliography.

Adelberg, E. A., Mandel, M. and Chen, G.C.C. (1965). Optimal conditions for mutagenesis by N-Methyl-N'-Nitro-N-Nitrosoguanidine in *Escherichia coli* K12. *Biochem. Biophys. Res. Communi* . 18: 788-795.

Amy, N. K. and Rajagopalan, K.V. (1979). Characterisation of Molybdenum cofactor from *Escherichia coli. J. Bacteriol.* 140: 114-124.

Arst, H. N., Parbtani, A. A. M. and Cove, D. J. (1975). A mutant of *Aspergillus nidulans* defective in NAD-linked glutamate dehydrogenase. *Molec. Gen. Genet.* 138: 165-171.

Arst, H. N. Jr., Rand, K. N. and Bailey, C. R. (1979). Do the tightly linked structural genes for nitrate and nitrite reductases in *Aspergillus nidulans* form an Operon? Evidence from an insertional translocation which separates them. *Molec. Gen. Genet.* 17: 89-100.

Arst, H. N. Jr. and Page M. M. (1973). Mutants of *Aspergillus nidulans* altered in the transport of methylammonium and ammonium. *Molec. Gen. Genet.* 121: 239-245.

Arst, H. N. Jr. and MacDonald, D. W. (1973). A mutant of *Aspergillus nidulans* lacking NADP-linked glutamate dehydrogenase. *Molec. Gen. Genet.* 122: 261-265.

Arst, H. N. jr. (1968). Genetic analysis of the first steps of sulphate metabolism in *Aspergillus nidulans*. *Nature*. 219: 268-270.

Arst, H. N. jr. and Cove, D. J. (1969). Methylammonium resistance in *Aspergillus nidulans*. *J. Bacteriol.* 98: 1284-1293.

Arst, H. N. jr. and Cove, D. J. (1970). Molybdate metabolism in *Aspergillus nidulans*, I. Mutations affecting phosphate activity or galactose utilisation. *Molec.Gen.Genet* 108: 146-153.

Arst, H. N. jr., MacDonald, D. W. and Cove, D. J. (1970). Molybdate metabolism in *Aspergillus nidulans*.. I Mutations affecting Nitrate reductase and/or Xanthine dehydrogenase. *Molec.Gen.Genet.* 108: 129-145.

Arst, H. N. jr. and Cove, D. J. (1973). Nitrogen metabolite repression in *Aspergillus nidulans*. *Molec.Gen.Genet.* 126: 111-141.

Arst, H. N. jr. (1981). Aspects of the control of gene expression in fungi. *Symp. Soc.Gen. Microbiol.* 31: 131-160.

Arst, H. N. jr., Jones, S. A. and Bailey, C. R. (1981). A method for the selection of deletion mutations in the L-proline catabolism gene cluster of *Aspergillus nidulans*. *Genet. Res.* 38: 171-195.

Arst, H. N. jr., Tollervey, D. W. and Sealy-lewis, H. M. (1982). A possible regulatory gene for the Molybdenum-containing cofactor in *Aspergillus nidulans*. *J. Gen. Microbiol.* 128: 1083-1093.

Arst, H. N. Jr. (1984). Regulation of gene expression in *Aspergillus nidulans*. *Microbiol. Sci.* 1: 137-140.

Arst, H. N. Jr. (1983). Fungal systems. In eukaryotic genes: Their structure, activity, and regulation. Edited by Maclean, N., Gregory, S. G. and Flavell, R. A. *Butterworths, London.* pp 433-450.

Back, E., Dunne, W., Scchneiderbauer, A., de Framont, A., Rastogi, A. and Rothstein, S. J. (1991). Isolation of the spinach nitrite reductase gene promoter which confers nitrate inducibility on GUS gene expression in transgeneic tobacco. *Plant. Mol. Biol.* 17: 9-18.

Ballance, D. J. and Turner, G. (1985). Development of a high-frequency transforming vector for *Aspergillus nidulans*. *Gene.* 36: 321-331.

Bamforth, F. J., Johnson, J. L., Davidson, A. G. F., Wong, L. T. K., Lockitch, G. and Applegarth, D. A. (1990). Biochemical investigation of a child with Molybdenum cofactor Deficiency. *Clin Biochem.* 23: 537-542.

Brody, H. and Carbon, J. (1989). Electrophoretic karyotype of *Aspergillus nidulans. Proc. Nat. Acad. Sci. (USA)* . 86: 6260-6263.

Brody, H., Griffith, J., Cuticchia, A. J., Arnold, J. and Timberlake, W. E. (1991). Chromosome-specific recombinant DNA libraries from the fungus *Aspergillus nidulans. Nucl. Acid. Res.* 19: 3105-3109.

Brownlee, A. G. and Arst, H. N. jr. (1983). Nitrate uptake in *Aspergillus nidulans* and the involvement of the third gene of the nitrate assimilatory gene cluster. *J. Bacteriol.* 115: 1138-1146.

Burger, G., Strauss, J., Scazzocchio, C. and Lang, B. F. (1991 a). *nirA*, the pathway-specific regulatory gene of nitrate assimilation in *Aspergillus nidulans*, encodes a putative GAL4-type zinc finger protein and contain four introns in highly conserved regions. *Mole. Cell. Biol.* 11: 5746-5755.

Burger, G., Tilburn, J. and Scazzocchio, C. (1991 b). Molecular cloning and functional characterisation of the pathway specific regulatory gene *nirA*, which controls nitrate assimilation in *Aspergillus nidulans. Mole. Cell. Biol.* 11: 795-802.

Caboche, M., Cherel, L., Galangau, F., Grandbastian, M. A., Meyer, C., Moureaux, T., Pelsy, F., Rouze, P., Vaucheret, H., Vedele, F. and Vincentz, M. (1989). Molecular genetics of nitrate reduction in *Nicotiana*. In: Molecular and genetic aspects of nitrate assimilation. Edited by Wray, J. L. and Kinghorn, J. R. *Oxford University Press, Oxford.*. pp 186-190.

Campbell, W. H. and Kinghorn, J. R. (1990). Functional domains of assimilatory nitrate reductases and nitrite reductases. In: Trends in Biochemical science. *Elsevier Science Publishers.(UK).* 15: pp 315-319.

Caroline, D., Christina, S., Tania, P., Jonathane, J. and Clare, L. (1992). Behaviour of the maize transposable element AC in *Arabidopsis thaliana*. *Plant J.* 2: 69-81.

Cole, J. A. (1989). Physiology, biochemistry, and genetics of nitrite reduction by *Escherichia coli*.. In: Molecular and Genetic aspects of Nitrate Assimilation. Edited by Wray, J. L. and Kinghorn, J. R. *Oxford University Press, Oxford.* PP. 229-243

Clutterbuck, A. J. (1994). *Aspergillus nidulans* linkage map. In: *Aspergillus*: 50 years on. Edited by Martinelli, S. D and Kinghorn, *J. R. Elsevier, Amsterdam.* 29.

Cooley, N. R. and Tomsett, B. A. (1985.). Determination of the subunit size of NADPH nitrate reductase from *Aspergillus nidulans*. *Biochim. Biophys. Acta.* 831: 89-93.

Cordoba, F., Cardenas, J. and Fernandez, E. (1986). Kinetic characterisation of nitrite uptake and reduction by *Chlamydomonas reinhardtii*. *Plant Physiol.* 82: 904-908.

Cornwell, E. V. and. MacDonald., D. W. (1984). *glnA* mutations define the structural gene for glutamine synthetase in *Aspergillus nidulans*. *Curr. Genet.* 8: 33-36.

Cove, D. J. and Pateman, J. A. (1963). Independently segregating loci concerned with nitrate assimilation in *Aspergillus nidulans*. *Nature.* 168: 262-263.

Cove, D. J. (1966). The induction and repression of nitrate Reductase in the fungus *Aspergillus nidulans*. *Biochem. Biophys. Acta.* 113: 51-56.

Cove, D. J. (1967). Kinetic studies of the induction of Nitrate Reductase and Cytochrome-C reductase in the fungus *Aspergillus nidulans*. *Biochem. J.* 104: 1033-1039.

Cove, D. J. (1969). Autoregulation of the synthesis of NiR in *A.nidulans*. *J. Bacteriol.* 97: 1374-1378.

Cove, D. J. (1970). Control of gene action on *Aspergillus nidulans*. *Proc. R. Soc. Lond.* 176: 267-275.

Cove, D. J. (1976). Chlorate Toxicity in *Aspergillus nidulans*, studies of mutants altered in Nitrate Assimilation. *Molec. Gen. Genet.* 146: 147-159.

Cove, D. J. (1976). Chlorate toxicity in *Aspergillus nidulans*: The selection and characterisation of chlorate resistant mutants. *Hered.* 36: 191-203.

Cove, D. J. (1979). Genetical studies of nitrate assimilation in *Aspergillus nidulans*. *Biol. Rev.* 54: 291-303.

Cove, D. J. and Coddington, A. (1965). Purification of nitrate reductase and Cytochrome-C reductase from *Aspergillus nidulans*. *Biochim. Biophys. Acta* 110: 312-318.

Cove, D. J. and Pateman, J. A. (1969). Autoregulation of the synthesis of nitrite reductase in *Aspergillus nidulans*. *J. Bacteriol.* 97: 1374-1378.

Cove, D. J., Pateman, J. A. and Rever, B. M. (1964). Genetic control of nitrate reduction in *Aspergillus nidulans*. *Hered.* 19: p529.

Crawford, N. M. (1995). Nitrate: Nutrient and signal for plant growth. *Plant Cell.* 7: 859-868.

Crawford, N. M., Smith, M., Bellissimo, D. and Davis, R. W. (1988). Sequence and nitrate regulation of the *Arabidopsis thaliana* mRNA encoding Nitrate Reductase, a metalloflavoprotein with three functional domains. *Proc. Nat. Acad. Sci. (USA).* 85: 5006-5010.

Dailey, F. A., Warner, R. L., Somers, D. A. and Kleinhofs, A. (1982). Characterisation of a Nitrate Reductase in a Barley mutant Deficient in NADH nitrate reductase. *Plant Physiol.* 69: 1200-1204.

Darlington, A. J., Scazzocchio, C. and Pateman, J. A. (1965). Biochemical and genetical studies of purine breakdown in *Aspergillus. Nature.* 206: 599-600.

Darlington, A. J. and Scazzocchio, C. (1968). Evidence for an alternative pathway of xanthine oxidation in *Aspergillus nidulans. Biochim. Biophys. Acta.* 166: 569-571.

Davis, M. A., Small, A. J., Kourambas, S. and Hynes, M. J. (1996). The *tamA* gene of *Aspergillus nidulans* contains a putative zinc cluster motif which is not required for gene function. *J. Bacteriol.* 178: 3406-3409.

Dean, C., Sjodin, C., Page T., Jones, J. and Lister, C. (1992). Behaviour of the maize transposable element Ac in *Arabidopsis thaliana. Plant J.* 2: 69-81.

Doddema, H. and Telkamp, G. P. (1979). Uptake of nitrate by mutants of *Arabidopsis thaliana*, disturbed in uptake or reduction of nitrate. *II. kinetics, Physiol. Plant.* 45: 332-338.

Downey, R. J. and Focht, W. J. (1974). The subunit size of *Aspergillus nidulans* nitrate reductase. *Microbios.* 11: 61-70.

Downey, R. J. and Steiner, F. X. (1979). Further characterisation of the reduced nicotinamide adenine dinucleotide phosphate: nitrate oxireductase in *Aspergillus nidulans. J. Bacteriol.* 137: 105-114.

Dunn-Coleman, N. S. and Smarrelli, J. Jr. and Garrett R. H. (1984). Nitrate assimilation in eukaryotic cells. *Inter. Rev Cytol.* 92: 1-50.

Ferguson, A. R. (1969). The nitrogen metabolism of *spirodela oligorriza.* II. Control of the enzymes of nitrate assimilation. *Planta.* 88: 353-363.

Ferguson, A. R. and Bollard, E. G. (1969). Nitrogen metabolism of *Spirodela oligorriza.* I.Utilisation of ammonium, nitrate, and nitrite. *Planta.* 88: 344-352.

Fincham, J. R. S. (1989). Transformation in fungi. *Microb. Rev.* 53: 148-167.

Forman, D. and Shunker, D. E. G. (1989). Nitrite and nitroso compounds in human cancer. In Cancer Surveys. *Cold Spring Harbour Press, (USA).*

Fu, Y.-H. and. Marzluf., G. A. (1990 a). *nit-2*, the major nitrogen regulatory gene of *Neurospora crassa*, encodes a protein with a putative zinc finger DNA-binding domain. *Mole. cell. Biol.* 10: 1056-1065.

Galvan, A., Quesada, A. and Fernandez, E. (1996). Nitrate and nitrite are transported by different specific transport systems and by a bispecific transporter in *Chlamydomonas reinhardtii. J. Biol. Chem.* 271: 2088-2092.

Garde, J., Kinghorn, J. R. and Tomsett, A. B. (1995). Site-directed mutagenesis of nitrate reductase from *Aspergillus nidulans*: Identification of some essential and some non-essential amino acids among conserved residues. *J. Biol. Chem.* 270: 6644-6650.

Garret, R. H. and Nason, A. (1969). Further purification and properties of *Neurospora* nitrate reductase. *J. Biol. Chem.* 244: 2870-2882.

Garrett, R. H. and Cove, D. J. (1976). Formation of NADPH-nitrate reductase activity in vitro from *Aspergillus nidulans niaD* and *cnx* mutants. *Mole. Gen. Genet.* 149: 189-196.

Gems, D., Johnstone, I. L. and Clutterbuck, A. J. (1991). An autonomously replicating plasmid transforms *Aspergillus nidulans* at high frequency. *Gene.* 98: 61-67.

Gems, D., Aleksenko, A., Belenky, L., Robertson, S., Ramsden, M., Vinetski, Y. and Clutterbuck, A. J. (1994). An 'Instant gene bank' method for gene clonning by mutant complementation. *Molec. Gen. Genet* 242: 467-471.

Gibson, T. J., Coulson, A. R., Sulston, J. E. and Little, P. F. R. (1987). Lorist 2 a cosmid with transcriptional terminator insulating vector genes from interference by promoters within the insert: Effect on DNA yield and cloned insert frequency. *Gene.* 53: 275-281.

Glaser, J. H. and DeMoss., J. A. (1971). Phenotypic restoration by molybdate of nitrate reductase activity in *chlD* mutants of *Escherichia coli. J. Bacteriol.* 108: 854-860.

Glass, A. D., Shaff, J. E. and Kochian, L. V. (1992). Studies of the uptake of nitrate in barley. IV. Electrophysiology. *Plant Physiol.* 99: 456-463.

Goldsmith, J., Livoni, J. P., Norberg, C. L. and Segal, I. H. (1973). Regulation of nitrate uptake in *Penicillium chrysogenum* by ammonium ion. *Plant Physiol.* 52: 362-367.

Hawker, K. L., Montague, P. and Kinghorn, J. R. (1992). Nitrate reductase and nitrite reductase transcripts levels in various mutants of *Aspergillus nidulans*: confirmation of autogenous regulation. *Mol. Gen. Genet.* 231: 485-488.

Heck, I. S. and. Ninnemann., H. (1995). Molybdenum cofactor biosynthesis in *Neurospora crassa*: Biochemical characterisation of pleiotropic molybdenum mutants *nit-7*, *nit-8*, *nit-9A*, B, and C. *Photochem. Photobiol.* 61: 54-60.

Heimer, Y. M. and Filner, P. (1971). Regulation of the nitrate assimilation pathway in cultured tobacco cells. III. The nitrate uptake system. *Biochim. Biophys. Acta.* 230: 362-372.

Herman, C. and Clutterbuck, A. J. (1966). A method for selection of auxotrophs by means of spidery growth. *Aspergillus Newsletter.* 7: 13-14.

Hinton, S. M. and Dean, D. (1990). Biogenesis of molybdenum Co-factors. *CRC Rev. Microbiol.* 17: 169-188.

Hoff, T., Schnorr, K. M., Meyer, C. and Caboche, M. (1995). Isolation of two *Arabidopsis* cDNA's involved in early steps in molybdenum cofactor biosynthesis by functional complementation of *Echerichia coli* mutants. *J. Biol. Chem.* 270: 6100-6107.

Hole, D., Emran, A. M., Fares, Y. and Drew, M. C. (1990). Induction of nitrate transport in maize roots and kinetics of influx, measured with nitrogen-13. *Plant. Physiol.* 93: 642-647.

Horner, R. D. (1983). Purification and comparison of *nit-1* and wild-type nitrate reductase of *Neurospora crassa. Biochim. Biophys. Acta.* 744: 7-15.

Hynes, M. J. (1973). The effect of lack of a carbon source on nitrate reductase activity in *Aspergillus nidulans. Mol. Gen. Genet.* 79: 155-157.

Hynes, M. J. (1975). Transformation for filamentous fungi. *Aust. J. Biol. Sci.* 28: 301-313.

Hynes, M. J. (1979). Fine-structure mapping of the acetamidase structural gene and its controlling region in *Aspergillus nidulans. Genet.* 91: 381-392.

Hynes, M. J. (1986). Transformation of the filamentous fungi. *Experi. Mycol.* 10: 1-8.

Hynes, M. J. and Pateman, J. A. (1970a). The genetic analysis of regulation of amidase synthesis in *Aspergillus nidulans*. I. mutants unable to utilise acrylamide. *Mol. Gen. Genet.* 108: 97-106.

Johnson, J. L. (1980). The molybdenum cofactor common to nitrate reductase, xanthine dehydrogenase, and sulphite oxidase. In Molybdenum and molybdenum-containing enzymes. Edited by Coughlan, M. P. *Pergamon. Press, New York..* pp 345-383.

Johnson, J. L., Hainline, B. E. and Rajagopalan, K. V. (1984). The pterin component of the molybdenum cofactor. *J. Biol. Chem.* 259: 5414-5422.

Johnson, J. L., Wuebben, M. M., Mandell, R. and Shih, V. E. (1989). Molybdenum cofactor biosynthesis in humans, identification of two complementation groups of cofactor-deficient patients and preliminary characterisation of a diffusible molybdenum precursor. *J. Clin. Invest.* 83: 897-903.

Johnson, J. L., Indermaur, L. W. and Rajagopalan, K. V. (1991). Molybdenum cofactor biosynthesis in *Escherichia coli. J. Biol. Chem.* 266: 12140-12145.

Johnstone, I. L. (1985). Transformation in *Aspergillus nidulans. Microbiol. Sci.* 2: 307-311.

Johnstone, I. L., McCabe, P.C., Greaves, P., Gurr, S. J., Cole, G. E., Brow, M. A. D., Unkles, S. E., Clutterbuck, A. J., Kinghorn, J. R. and Innis, M. A. (1990). Isolation and characterisation of the *crnA-niiA-niaD* gene cluster for nitrate assimilation in *Aspergillus nidulans*. *Gene*. 90: 181-192.

Joshi, M. S., Johnson, J. L. and Rajagopalan, K. V. (1996). Molybdenum cofactor biosynthesis in *Escherichia coli mod* and *mog* mutants. *J. Bacteriol.* 178: 4310-4312.

Kamdar, K. P., Shelton, M. E. and Finnerty, V. (1994). The *Drosophila* molybdenum cofactor gene, Cinnamon is homologous to three *Escherichia coli* cofactor proteins and to the rat protein Gephyrin. *Genet.* 137: 791-801.

Kim, J. and Rees, D. C. (1994). Nitrogenase and Biological nitrogen fixation. *Biochem* 33: 389-397.

Kinghorn, J. R., and Campbell, E. I. (1989). Amino acid sequence relationships between bacteria, fungi, and plant nitrate reductase and nitrite reductase proteins. In Molecular and genetic aspects of nitrate assimilation. Edited by Wray, J. L. and Kinghorn, J. R. *Oxford. University. Press, Oxford.* pp 385-403.

Kinghorn, J. R., and Pateman, J. A. (1975). Studies of partially derepressed mutants at the *tamA* and *areA* loci in *Aspergillus nidulans*. *Mol. Gen. Genet.* 140: 137-147.

Kinghorn, J. R. and. Pateman., J. A. (1976). Mutants of *Aspergillus nidulans* lacking Nicotinamide Adenine Dinucleotide-specific glutamate dehydrogenase. *J. Bacteriol.* 125: 42-47.

Kinghorn, J. R. (1989). Genetical, biochemical, and structural organisation of the *Aspergillus nidulans crnA-niiA-niaD* gene cluster. In. Molecular and genetic aspects of nitrate assimilation. Edited by Wray, J. L. and Kinghorn, J. R. *Oxford University Press, Oxford,* pp 69-87.

Kinghorn, J R., Montague, P. and Unkles, S. E. (1990). Regulatory, structural, and biotechnological aspects of the *Aspergillus nidulans* gene cluster for nitrate assimilation. In. Inorganic nitrogen in plants and microorganisms; uptake and metabolism. Edited by Ullrich, W. R., Rigano, C., Fuggi, A., Aspricio, P. J. *Springer. Verlag. Press.*. pp 287-295.

Kinghorn, J. R. and Unkles, S. E. (1994). Inorganic nitrogen assimilation: Molecular aspects. In. *Aspergillus*: 50 years on. Edited by Martinelli, S. D. and Kinghorn, J. R. *Elsevier. Amsterdam.* 29: pp 181-194.

Kinghorn, J. R. and. Pateman, J. A. (1973). NAD and NADP L-glutamate dehydrogenase activity and ammonium regulation in *Aspergillus nidulans. J Gen Micobiol* 78: 39-46.

Kramer, S. P., Johnson, J. L., Ribeiro, A. A., Millington, D. S. and Rajagopalan, K. V. (1987). The structure of the molybdenum cofactor. Characterisation of DI-(carboxyamidomethyl) molybdopterin from sulphite oxidase and xanthine oxidase. *J. Biol. Chem.* 262: 16357-16363.

Kudla, B., Caddick, M. X., Langdon, T. (1990). The regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans.* Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. *EMBO. J.* 9: 1355-1364.

LaBrie, S. T., Wilkinson, J. Q., Tsay, Y. F. and Feldmann, K. A. (1992). Identification of two tungstate-sensitive molybdenum cofactor mutants, *chl2* and *chl7*, of *Arabidopsis thaliana. Mol. Gen. Genet.* 233: 169-176.

Leach, J., Finkelstein, D. B. and Rambosek, J. A. (1986). Rapid mini-prep of the DNA from filamentous fungi. *Neurospora Newsletter.* 33: 32-33.

Lewis, N. J., Hurt, P., Sealy-Lewis, H. M. and Scazzocchio, C. (1978). The genetic control of the molybdopterin in *Aspergillus nidulans* IV. A comparison between purine hydroxylase I and II. *Euro. J. Biochem..* 91: 311-316.

Lobbi-Nivol, C., Palmer, T., Whitty, P. W., McNairn, E. and Boxer, D. H. (1995). The *mob* locus of *Escherichia coli* K12 required for molybdenum cofactor biosynthesis is expressed at very low levels. *J. Microbiol* . 141: 1663-1671.

Low, D. C., Pommier, J., Giodano, G. and Boxer, D. H. (1988). Biosynthesis of molybdo-enzymes in *E. coli* : *chlB* is the only chlorate resistance locus required for protein FA activity. *FEMS Microbiol. Letters*. 49: 331-336.

Lyclama, J. C. (1963). The absorption of ammonium and nitrate by perenial rye-grass. *Acta Bot. Neerl.* 12: 316-423.

MacDonald, D. W. and Cove, D. J. (1974). Cytochrome-C Reductases from wild-type and mutant strains of *Aspergillus nidulans*. *Molec. Gen. Genet.* 128: 187-199.

MacDonald, D. W. and Cove, D. J. (1974). Studies on Temperature sensitive mutants affecting the assimilatory Nitrate Reductase of *Aspergillus nidulans*. *Eur. J. Biochem.* 47: 107-110.

MacDonald, D. W. and Cove, D. J. (1972). Studies on temperature sensitive *cnx* mutants in the fungus *Aspergillus nidulans*. *Biochem. J.* 127: 1-19.

MacDonald, D. W., Cove, D. J. and Coddington, A. (1974). Cytochrome-C reductase from wild-type and mutant strains of *Aspergillus nidulans*. *Molec. Gen. Genet.* 128: 187-199.

MacDonald, D. W. (1982). A single mutation leads to loss of glutamine synthetase and relief of ammonium repression in *Aspergillus nidulans*. *Curr. Genet.* 6: 203-208.

Martin, F. and Botton, B. (1993). Nitrogen metabolism of ectomycorrhizal fungi and ectomycorrhiza. *Adv. Plant. Pathol.* 9: 83-102.

Martinelli, S. D. (1994). *Aspergillus nidulans* as an experimental organism. In *Aspergillus*:: 50 years on. Edited by Martinelli, S. D. and Kinghorn, J. R. *Elsevier, Amsterdam.* 29: pp 33-58.

Maupin-Furlow, J. A., Rosentel, J. K., Lee, J. H., Deppenmeier, U., Gunsalm, R. P. and Shanmugam, K. T. (1995). Genetic analysis of the *modABCD* (molybdate transport) Operon of *Escherichia. coli. J. Bacteriol.* 177: 4851-4856.

Mehra, R. K. and Coughlan, M. P. (1984). Purification and properties of purine hydroxylase II from *Aspergillus nidulans*. *Arch Biochem Biophys.* 229: 585-595.

Meyerowits, E. M. (1994). Structure and organisation of the *Arabidopsis thaliana* Nuclear genome. In. *Arabidopsis.* Edited by Meyerowits, E. M. and Somerville, C. R. *Cold Spring Harbor Laboratory Press (USA).* ch 2: pp 21-36.

Minagawa, N. and Yoshimoto, A. (1982). Purification and Characterisation of the Assimilatory NADPH-Nitrate-Raductase of *Aspergillus. nidulans. J Biochem* 91: 761-779.

Minehart, P. L. and. Magasinik., B. (1991). Sequence and expression of GLN3, a positive nitrogen regulatory gene of *Saccharomyces cerevisiae* encoding a protein with a putative zinc finger DNA domain. *Mole. Cell. Biol.* 11: 6216-6228.

Mishra, N. C. (1985). Gene transfer in fungi. In: Advances in genetics. *Academic. Press.* 23: 73-173.

Okamoto, P. M. and Marzluf, G. A. (1993). Nitrate reductase of *Neurospora. crassa*: The functional role of individual amino acids in the haeme domain as examined by site-directed mutagenesis. *Molec Gen Genet* 240: 221-230.

Palmer, T., Santini, C-L., Lobbi-Nivol, C., Eaves, D. J., Boxer, D. H. and Giordano, G. (1996). Involvment of the *narJ* and *mob* gene products in distinct steps in the biosynthesis of the molybdoenzyme nitrate reductase in *Escherichia coli. Molec. Biol.* 20: 875-884.

Pan, S. S. and Nason, A. (1978). Purification and characterisation of homogeneous assimilatory reduced nicotinamide adenine dinucleotide phosphate-nitrate reductase from *Neurospora Crassa.. Biochim. Biophys. Acta.* 523: 297-313.

Pardee, A. B., Prestidge, L. S., Whipple, M. B. and Dreyfuss, J. (1966). A binding site for sulphate and its relation to sulphate transport in *Salmonella. typhimurium. J. Biol. Chem.* 241: 3962-3969.

Pateman, J. A. (1969). Regulation of the synthesis of glutamic dehydrogenase and glutamine synthetase in mico-organisms. *Biochem J.* 115: 769.

Pateman, J. A., Cove, D. J., Rever, B. M. and Roberts, D. B. (1964). A common Co-factor for nitrate reductase and xanthine dehydrogenase, which also regulates the synthesis of nitrate reductase. *Nature.* 201: 58-60.

Pateman, J. A., Kinghorn, J. R., Dunn, E. and Forbes, E. (1973). Ammonium regulation in *Aspergillus nidulans. J. Bacteriol.* 114: 943-950.

Pateman, J. A., Rever, B. M. and Cove, D. J. (1967). Genetical and biochemical studies of nitrate assimilation in *Aspergillus nidulans.. Biochem. J.* 104: 103-111.

Peberdy, J. F. (1991). Fungal protoplasts. In: more gene manipulations in fungi. *Acad. Press.* ch 14: pp 307-314.

Pelsy, F., Kronenberger, J., Pollien, J. M. and Caboche, M. (1991). M2 seed screening for nitrate reductase deficiency in *Nicotiana. plumbaginifolia. Plant. Sci.* 76: 109-114.

Pitterle, D. and Rajagopalan, K. V. (1993). In vitro synthesis of molybdopterin from precursor Z using purified converting factor. *J. Biol. Chem.* 268: 13506-13509.

Polley, S. D. and. Mark, X. Caddix. (1996). Molecular characterisation of *meaB*, a novel gene affecting nitrogen metabolite repression in *Aspergillus nidulans. FEBS Letters.* 388: 200-205.

Pombeiro, S. R. C., Martinez-Rossi, N. M. and Rossi, A. (1983). Nitrate toxicity in *Aspergillus nidulans*: a new locus in a *proA1 paba A6 yA2* strain. *Genet Res* 41: 203-207.

Pontecorvo, G., Roper, J. A., Hemmons, L. H., MacDonald, K. D., Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans. Adv Genet.* 5: 141-238.

Prodouz, K. N. and Garrett, R. H. (1981). *Neurospora crassa* NAD(P)H-nitrate reductase. Studies of its composition and structure. *J. Biol. Chem* . 256: 1971-1981.

Quesada, A., Galvan, A. and Fernandez, E. (1994). Identification of nitrate transporter genes in *Chlamydomonas reinhardtii. Plant. J.* 5: 407-419.

Rajagopalan, K. V. (1989). Chemistry and biology of molybdenum cofactor. In. Molecular and genetic aspects of nitrate assimilation. Edited by Wray, J. L. and Kinghorn, J. R. *Oxford University. Press, Oxford.* ch 14: pp 212-226.

Rajagopalan, K. V. and Jonhson, J. L. (1992). The pterin Molybdenum cofactors. *J. Biol. Chem* . 267: 10199-10202.

Ramaiah, A. and Shanmugasundaram., E. R. B. (1962a). Effect of molybdenum toxicity on sulphur metabolism in *Neurospora. crassa. Biochim. Biophys. Acta.* 60: 373-385.

Raman, N., Sivarama, S. K. and Sarma, P. S. (1962). The influence of sulphur compounds on molybdate toxicity in *Aspergillus nigar. Biochim. Biophys. Acta.* 56: 195-197.

Ramaswamy, K. S., Endley, S. and Golden, J. W. (1996). Nitrate reductase activity and hetercyst suppression on nitrate in *Anabaena* sp. strain PCC 7120 require *moeA. J. Bacteriol.* 178: 3893-3898.

Rech, S., Deppenmeier, U. and Gunsalus, R. P. (1995). Regulation of the molybdate transport Operon, *modABCD* of *Escherichia coli* in response to molybdate availability. *J. Bacteriol.* 177: 1023-1029.

Riach, M. B. R. and Kinghorn, J. R. (1996). Genetic transformation and vector development in filamentous fungi. In. Fungal genetics: principles and practice. Edited by Bos, C. *J. Marcel Dekker, New York* 13: pp 209-233.

Rivers, S. L., McNairn, E., Blasco, F., Giordano, G. and Boxer, D. H. (1993). Molecular-genetic analysis of the *moa* Operon of *Escherichia coli* K12 required for molybdenum cofactor biosynthesis. *Molec. Microbiol.* 8: 1071-1081.

Roberts, C. F. (1963). The genetics of carbohydrate utilisation in *Aspergillus nidulans. J. Gen. Microbiol.* 31: 45-48.

Rosentel, J. K., Healy, F., Maupin-Furlow, J. A., Lee, J. H. and Shanmugam, K. T. (1995). Molybdate and regulation of *mod* (molybdate transport), *fdhF* and *hyc* (formate dehydrogenase) Operons in *Escherichia. coli. J. Bacteriol.* 177: 4857-4864.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual. *Cold Spring Harbor Laboratory Press, (USA).*

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-termination inhibitors. *Proc. Nat. Acad. Sci. (USA).* 74: 5463-5467.

Santini, C. L., Labbi-Nivol, C., Romane, C., Boxer, D. H. and Giodana, G. (1992). Molybdoenzyme biosynthesis in *Escherichia. coli* : in vitro activation of purified nitrate reductase from a *chlB* mutant. *J. Bacteriol.* 174: 7934-7940.

Saunders, G., Michael, F. T. and Geoffrey, H. (1986). Fungal clonning vectors. *Elsevier Publishers.*

Scazzocchio, C. (1973). The genetic control of Molybdoflavoproteins in *Aspergillus nidulans.* II. Use of the NADH dehydrogenase activity associated with xanthine dehydrogenase to investigate substrate and product induction. *Molec. Gen. Genet.* 125: 147-155.

Scazzocchio, C. (1974). The genetic determination of molybdoflavoenzymes in *Aspergillus nidulans. J. of the less-common metals.* 36: 461-464.

Scazzocchio, C. (1980). The genetics of the Molybdenum containing enzymes. In Molybdenum and Molybdenum-containing enzymes. Edited by Coughlan, M. P. *Pergamon. Press, Oxford.* pp 487-515.

Scazzocchio, C. and Arst, H. N. Jr. (1989). Regulation of nitrate assimilation in *Aspergillus nidulans.* In. Molecular and genetic aspects of nitrate assimilation. Edited by Wray, J. L. and Kinghorn, J. R. *Oxford. University. Press, Oxford.* ch 19: pp 299-313.

Scazzocchio, C., Holl, F. B. and Foguelman, A. I. (1973). The genetic control of molybdoflavoproteins in *Aspergillus nidulans.* Allopurinol-resistant mutants constitutive for xanthine dehydrogenase. *Eur. J. Biochem.* 36: 428-445.

Schloemer, R. H. and Garrett, R. H. (1974). Nitrate transport system in *Neurospora crassa. J. Bacteriol.* 118: 259-269.

Shen, T. C., Funkhouse, E. A. and Guerrero, M. G. (1976). NADH-and NAD(P)H-nitrate reductase in rice seedlings. *Plant. Physiol.* 68: 292-294.

Snell, F. D. and Snell, C. T. (1949). Nitrites. In Colorimetric methods of analysis. *D. Van Nostrand Company, Inc, Toronto.* 2: pp 802-807.

Solomonson, L. and. McCeery., M. J. (1986). Radiation inactivation of assimilatory NADH: nitrate reductase from *Chlorella.* Catalytic and physical sizes of functional units. *J. Biol. Chem.* 261: 806-810.

Sophianopoulou, V. and Diallinas, G. (1995). Amino acid transporters of lower eukaryotes: regulation, structure, and topogenesis. *FEMS. Microbiol. Rev.* 16: 53-75.

Stallmeyer, B., Nerlich, A., Schiemann, J., Brinkmann, H. and Mendel, R. R. (1995). Molybdenum Co-factor biosynthesis: the *Arabidopsis thaliana* cDNA *cnx1* encodes a multifunctional two-domain protein homolougus to a mammalian neuroprotein, the insect protein Cinnamon and three *Escherichia coli* proteins. *Plant. J.* 8: 101-112.

Steiner, F. X. and Downey, R. J. (1982). Isoelectric focussing and two-dimentional analysis of purified Nitrate Reductase. *Biochim. Biophys. Acta.* 706: 203-211.

Syrett, P. J. and Morris, I. (1963). The inhibtion of nitrate assimilation by ammonium in *Chlorella. Biochim. Biophys. Acta.* 67: 560-575.

Tilburn, J., Scazzocchio, C., Taylor, G. G., Zabicky-Zissman, J. H., Lockington, R. A. and Davies, R. W. (1983). Transformation by integration in *Aspergillus nidulans. Gene.* 26: 205-221.

Timberlake, W. E. and Clutterbuck, A. J. (1994). Genetic regulation of conidiation. In Aspergillus 50 years on. Edited by Martinnelli, S. D. and Kinghorn, J. R. *Elsevier. Amsterdam.* 29: pp 383-427.

Timberlake, W. E. (1980). Developmental gene regulation in *Aspergillus nidulans.. Devel. Biol.* 78: 497-510.

Timberlake, W. E., Marshal, M. A. (1989). Genetic engineering of filamentous fungi. *Science.* 244: 1313-1317.

Timberlake, W. E., Boylan, M. T., Cooley, M. B., Mirabito, P. M., O'hara, E. B. and Willett, C. E. (1985). Rapid identification of mutation-complementing restriction fragments from *Aspergillus nidullans* cosmids. *Exper. Mycol.* 9: 351-355.

Tomsett, A. B. and Garrett, R. H. (1980). The isolation and characterisation of mutants defective in nitrate assimilation in *Neurospora crassa. Genet* 95: 649-660.

Tomsett, B. A. and Cove, D. J. (1979). Deletion mapping of the *niiA-niaD* gene region of *Aspergillus nidulans. Genet. Res.* 34: 19-32.

Tomsett, B. A. and. Garrett, R. H. (1980). The isolation and characterisation of mutants defective in nitrate assimilation in *Neurospora crassa. Genet.* 95: 649-660.

Tomsett, B. (1991). Nitrate assimilation in ascomycete fungi. In . Nitrogen phosphorus and sulphur utilisation by fungi. Edited by Boddy, L., Merchant, R. and Read, D. J. *Cambridge Press*, pp. 33-58.

Trueman, L. J., Richardson, A. and Forde, B. G. (1996). Molecular cloning of higher plant homologues of the high affinity nitrate transporters of *Chlamydomonas reinhardtii* and *Aspergillus nidulans. Gene* . 175: 223-231.

Tsay, Y. F., Schroeder, J. I., Feldmann, K. A. and Crawford, N. M. (1993). The herbicide sensitivity gene *chl1* of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. *Cell.* 72: 505-713.

Tweedie, J. W. and Segel, I. H. (1970). Specificity of transport processes for sulphur, selenium, and molybdenum anions by filamentous fungi. *Biochim. Biophys. Acta.* 196: 95-106.

Unkles, S. E. (1989). Fungal biotechnology and the nitrate assimilation pathway. In. Molecular and genetic aspects of nitrate assimilation. Edited by Wray, J. L and Kinghorn, J. R. *Oxford University. Press, Oxford.* pp 341-363.

Unkles, S. E., Campbell, E. I., Carrez, D., Grieve, C., Contreras, R., Fiers, W., Van Den Hondel, C. A. M. J. J. and Kinghorn, J. R. (1989). Transformation of *Aspergillus niger* with the homologous nitrate reductase gene. *Gene.* 78: 157-166.

Unkles, S. E., Hawker, K. L., Grieve, C., Campbell, E. I., Montague, P. and Kinghorn, J. R. (1991). *crnA* encodes a nitrate transporter in *Aspergillus nidulans. Proc. Nat. Acad. Sci (USA).* 88: 204-208.

Wadman, S. K., Duran, M., Beemer, F. A., Cats, B. P., Johnson, J. L., Rajagopalan, K. V., Saudubray, J. M., Ogier, H., Charpentier, C., Berger, R., Smith, G. P. A., Wilson, J. and Krywawych, S. (1983). Absence of hepatic molybdenum cofactor: An inborn error of metabolism leading to a combined deficiency of suphite oxidase and xanthine dehydrogenase. *J. Inher. Metab. Dis.* 1: 78-83.

Wahl, G. M., Lewis, K. A., Ruiz, J. C., Rothenberg, B., Zhao, J. and Evans, G. A. (1987). Cosmid vectors for rapid genomic walking, restriction mapping and gene transfer. *Proc. Nat Acad. Sci. (USA).* 84: 2160-2164.

Wray, J. L. and Kinghorn, J. R. (1989). Molecular and genetic aspects of nitrate assimilation. *Oxford. University. Press.*, Oxford.

Wray, J. L. and Filner, P. (1970). Structural and functional relationships of enzyme activities induced by nitrate in Barley. *Biochem. J.* 119: 715-725.

Wuebbens, M. M. and Rajagopalan, K. V. (1995). Investigation of the early steps of molybdopterin biosynthesis in *Escherichia coli* through the use of in vivo labelling studies. *J. Biol. Chem.* 270: 1082-1087.

Yamamoto, L. A. and Segel, I. H. (1966). The inorganic sulphate transport system of *penicillium chrysogeum.*. *Arch. Biochem.* 144: 523-538.

Yuan, G.-F., Fu, Y. -H. and Marzluf, G. A. (1991). *nit-4*, a pathway-specific regulatory gene of *Neurospora crassa*, encodes a protein with a putative binuclear zinc DNA-binding domain. *Mole. Cell. Biol.* 11: 5735-5745.